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DISTRIBUTION OF LIPIDS IN THE CENTRAL NERVOUS SYSTEM OF SOME VERTEBRATES

By YUKIO KAWAKITA

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(Received for publication, December 7, 1955)

The distribution of the lipids in the central nervous system of animals, has hitherto been described by a number of workers: Fränkel and Linnert (*1*) and Koch and Koch (*2*) examined the total lipids of the brain in different kinds of animals; later, the various lipids of the brain were exhaustively analysed by Kaucher, Garbraith, Button and William (*3*) in the ox, by Erickson, Avrin, Teague and William (*4*) in the dog, by William, Garbraith, Kaucher, Moyer, Allen and Macy (*5*) in the rat and by Johnson, McNabb and Rossiter (*6*) in several animals.

However, the nervous tissue examined by these investigators was restricted in most cases to that of the cerebrum or of the whole brain and the concentration reported at which each lipid was found in the tissue was generally fluctuated, probably because of the diversity of the analytical method employed.

In our laboratory, Rin (*7*), Fukuyama (*8*) and Mihara (*9*) have studied the concentration of the various lipids found in the central nervous system not only in mammals but also in some lower vertebrates, and further made a comparative study of the lipid contents of the four different parts of the nervous system, the cerebrum, cerebellum, brain stem and spinal cord.

The present work was undertaken to determine the amounts of cholesterol, cerebroside and phospholipids lecithin, cephalin and sphingomyelin) contained in the above four parts as well as in the whole brain in the following animals, and particularly more reliable methods were employed on the greater number of cases.

a) Mammals: The ox and rabbit.
b) Avians: The white leghorn.
c) Reptilians: The striped snake.

d) Amphibians : The frog (*Rana nigromaculata nigromaculata* (*Hallowell*)).

e) Fish : The mackerel.

EXPERIMENTAL

Material—The brain and the spinal cord were removed immediately after the animals were killed. In the ox, rabbit and hen, the brain was separated into the cerebrum, cerebellum and brain stem, and the ox cerebrum was further divided into the white and the grey matters. These materials were ground up in a mortar, and a small portion of the lump was kept cool with dry ice and acetone and dried *in vacuo* (10^{-3} mm. Hg). The brains of the animals, 5 frogs, 10 snakes and 3 mackerels were ground at once to obtain the sufficient amount of material for the following analysis.

Extraction of Lipids—The materials were treated by Bloor's (*10*) method modified by Johnson, McNabb and Rossiter (*6*). Samples, each 100–200 mg. in weight, were extracted successively with 20 ml. of ethanol in 2 portions, with 50 ml. of ethanol-ether (1:1) in 5 portions and with 40 ml. of ether in 2 portions, at temperature ranging between 30°–60° according to the boiling point of these solvents. Each extract obtained was filtered in turn through filter paper into a volumetric flask, and ether was added so as to bring up the total volume to 200 ml. An aliquot of 125 ml. of this extract was evaporated at 60° just to dryness under reduced pressure and in N_2 atmosphere. The residue was extracted successively with 15 ml. of petroleum ether (b.p. 40°–60°) in 5 portions, and then evaporated down to one ml. under reduced pressure at 60°.

Phospholipid was precipitated by adding 30 ml. of acetone and 20 drops of a saturated solution of $MgCl_2$ in ethanol. After centrifugation, the supernatant fluid was decanted into a 50 ml. volumetric flask and the flask filled up with acetone. The precipitate was dissolved with 5 ml. of methanol-ether (1:1), transferred quantitatively into volumetric flask and the entire volume made up to 25 ml. with the solvent.

Sample for the estimation of cerebroside was taken from the original ethanol-ether extract, one for that of cholesterol from the acetone-soluble material and another for that of phospholipids from the methanol-ether extract.

Cerebroside—Cerebroside was estimated by the method of Brand and Sperry (*11*). An aliquot of 50 ml. of the original ethanol-ether extract was hydrolyzed with 6 *N* HCl at 100° for 45 minutes. The galactose liberated on the hydrolysis was estimated cerimetrically by the method of Miller and Van Slyke (*12*) in which the *o*-phenanthroline ferrous complex was used as an indicator.

Total Cholesterol—Total cholesterol was estimated by Schoenheimer and Sperry's (*13*) method slightly modified by Sobel and Mayer (*14*). The color was read in a Hitachi electrophotometer at 620 mμ.

Phospholipids—The procedure adopted for the determination of the various phospholipids was the same as that described by Hack (*15*). It is based on the observation that the monoaminophospholipids, lecithin and cephalin, are completely hydrolyzed by *N* KOH in 18 hours at 37°. Whereas, under these conditions, no hydrolysis of sphingomyelin occurs. The total acid-soluble P and choline in the hydrolysate are a measure of the monoaminophospholipid and lecithin respectively (Schmidt, Benotti, Hershman and Thannhauser (*16*), Hack (*15*)). The difference between the total P and the acid-soluble P in the hydrolysate is a measure of the sphingomyelin, and the difference between the molecular equivalents of monoaminophospholipid and choline gives the molecular equivalents of cephalin.

For the estimation of total P, 1 ml. of the methanol-ether extract was oxidised under wet condition with 60 per cent perchloric acid (Furukawa, Nosue and Yoshikawa (*17*)), and added with ammonium molybdate and ammonium vanadate (Barton (*18*)). The color was read in a Hitachi electrophotometer at 420 mμ. Total phospholipid was calculated on the basis of

$$\text{total phospholipid} = \text{total lipid P} \times 25.$$

Monoaminophospholipid was estimated in the remaining 24 ml. of the methanol-ether solution which was evaporated just to dryness under reduced pressure at 60°. To the residue was added 5 ml. of *N* KOH and the mixture hydrolyzed at 37° for 18 hours. To an aliquot of 0.5 ml. of this solution was added 0.1 ml. of 5 *N* HCl and 3 ml. of 10 per cent trichloracetic acid and kept at room temperature for 1–2 hours. After centrifugation and filtration the total P of this filtrate was estimated by the method described above. After correction had been made for the dilutions, monoaminophospholipid was calculated on the basis of

$$\text{monoaminophospholipid} = \text{total P of trichloracetic acid filtrate} \times 25.$$

Lecithin was estimated by determining choline in the hydrolysate by the essential method of Glick (*19*). 3.0 ml. of the hydrolysate was neutralized with glacial acetic acid with thymolphthalein in ethanol as an indicator. After filtration, the filtrate was added with 2 ml. of 2 per cent ammonium reineckate dissolved in 0.5 *N* HCl, and kept in a refrigerator overnight. The precipitated choline reineckate was collected and washed with ethanol and dissolved in acetone. The color density was measured in a Beckman spectrophotometer at 526 mμ. After correction had been made for the dilutions, the lecithin was determined by calculation on the basis of

$$\text{lecithin} = \text{choline (as choline chloride) in the trichloracetic acid filtrate.} \times 5.56$$

Sphingomyelin and cephalin were estimated as follows :

$$\text{Sphingomyelin} = \text{total phospholipid} - \text{monoaminophospholipid}$$
$$\text{Cephalin} = \text{monoaminophospholipid} - \text{lecithin}$$

RESULTS

The results obtained are summarized in Tables I–V. In Table VI, the various lipid fractions are given in percentage to the " essential lipid " introduced by Johnson, McNabb and Rossiter (*6*), *i.e.* in percentage to the sum of total phospholipid, cholesterol and cerebroside.

TABLE I

Distribution of Lipids in the Cerebrum

Species	No. of case	Phospholipids				Total cholesterol	Cerebroside
		Total	Lecithin	Cephalin	Sphingomyelin		
Ox	3	23.03±0.51	3.53±0.22	15.87±0.34	3.27±0.15	7.70±0.14	9.67±0.56
Rabbit	3	23.33±0.29	3.17±0.14	17.20±0.47	2.97±0.35	7.87±0.39	9.13±0.49
Hen	3	18.60±0.25	3.26±0.15	12.26±0.28	3.07±0.48	7.50±0.01	6.26±0.22
Snake	5	13.62±0.25	4.18±0.09	8.30±0.23	1.34±0.16	5.10±0.16	
Frog	5	14.90±0.59	5.00±0.09	7.90±0.35	1.58±0.37	8.10±0.15	7.66±0.46
Mackerel	5	22.20±0.64	7.30±0.20	10.14±0.49	4.36±0.46	3.58±0.14	9.12±0.29

(expressed in mg./ 100 mg. of dry tissue with probable error.)

TABLE II

Distribution of Lipids in the Cerebellum

Species	No. of case	Phospholipids				Total cholesterol	Cerebroside
		Total	Lecithin	Cephalin	Sphingomyelin		
Ox	3	19.40±0.14	5.57±0.09	10.97±0.33	2.87±0.16	9.13±0.30	7·73±0.15
Rabbit	3	19.67±0.48	2.87±0.24	12.27±0.39	4.53±0.24	8.83±0.22	8.43±0.24
Hen	3	19.16±0.49	3.10±0.07	13.93±0.32	2.13±0.31	6.63±0.27	7.83±0.23

(expressed in mg./ 100 mg. of dry tissue with probable error.)

TABLE III

Distribution of Lipids in the Brain Stem

Species	No. of case	Phospholipids				Total cholesterol	Cerebroside
		Total	Lecithin	Cephalin	Sphingomyelin		
Ox	3	20.83±0.41	6.60±0.16	10.37±0.67	4.20±0.19	6.80+0.44	8.40±0.31
Rabbit	3	23.53±0.24	3.00±0.17	16.33±0.21	4·20±0.17	9.20±0.38	9.40±0.23
Hen	3	20.83±0.22	2.37±0.14	14.17±0.26	4.17±0.29	7.67±0.17	10.27±0.29

(expressed in mg./ 100 mg. of dry tissue with probable error.)

TABLE IV

Distribution of Lipids in the Spinal Cord

Species	No. of case	Phospholipids				Total cholesterol	Cerebroside
		Total	Lecithin	Cephalin	Sphingomyelin		
Ox	3	24.50±0.58	7.80±0.27	11.43±0.38	5.27±0.21	7.17±0.16	9.63±0.29
Rabbit	3	26.33±0.49	3.13±0.02	16.47±0,28	6.73±0.16	10.27±0.24	10.90±0.12
Hen	3	23.50±0.34	3.83±0.27	15.37±0.61	4.67±0.18	10.20±0.22	11.37±0.35

(expressed in mg./ 100 mg. of dry tissue with probable error.)

TABLE V

Comparison in Lipid Content between the White Matter and the Grey Matter in Ox Cerebrum

Material	No. of case	Phospholipids				Total cholesterol	Cerebroside
		Total	Lecithin	Cephalin	Sphingomyelin		
White	3	27.59±0.41	3.32±0.14	19.24±0.12	5.03±0.21	10.85±0.72	14.11±0.07
Grey	3	17.32±0.38	3.16±0.02	9.44±0.50	3.72±0.29	3.16±0.16	4.27±0.22

(expressed in mg./100 mg. of dry tissue with probable error.)

TABLE VI

Relative Concentration of Lipids in the White Matter and in the Grey Matter of Ox Cerebrum

Material	No. of case	Phospholipids				Total cholesterol	Cerebroside
		Total	Lecithin	Cephalin	Sphingomyelin		
White	3	50.8	6.0	35.4	9.0	20.0	26.0
Grey	3	66.9	12.2	36.5	14.3	12.2	16.5

(expressed in % to " Essential Lipid " *i.e.* the sum of total phospholipid, total cholesterol and cerebroside.)

DISCUSSION

In the case of brain tissue, the extraction procedure employed proved satisfactory as has been reported by Johnson, McNabb and Rossiter (*6*). After treatment by this method, the insoluble residue was extracted for 6 hours with ether in a continuous extraction apparatus, followed by a further 6 hours extraction with methanol. The combined extract was found to contain a negrigible quantity of phospholipid but not cerebroside or cholesterol at all.

There are many reports on the distribution of the lipids in the brain of the ox and rabbit, and the present findings that among three phospholipids—lecithin, cephalin and sphingomyelin—cephalin is contained in the greatest concentration in the materials examined is analogous to the result reached on the subject by Johnson, McNabb and Rossiter (*6*) and other workers.

As far as the author has known, a comparative study of the four parts of the central nervous system (*i.e.* cerebrum, cerebellum, brain stem and spinal cord) in respect of their lipid contents has very seldom been reported in the literature. In view of this fact Mihara (*9*) and Fukuyama (*8*) have studied the problem in some mammals, in the dog and in particular, Mihara (*9*) extending the field of his study even to a few lower vertebrates.

The tables given here and those published by Mihara (*9*), will considerably be varied especially in the results obtained in lower vertebrates, seemingly due in part to the differences of analytical method employed. But the two different tables are agreed on the following points regarding mammals:

1) In the rabbit and hen, cholesterol was found in the highest concentration in the spinal cord, in lower concentration in the brain stem and in the least concentration in the cerebellum.

2) Cerebroside was of the same distribution pattern as cholesterol in the rabbit and hen.

3) Total lipids and each constituents, with the exception of lecithin, were found in a larger amount in the white matter than in the grey. This finding was in confirming the conclusion reached by Johnson, McNabb and Rossiter (*6*) and by Schmidt, Benotti, Hershman and Thannhauser (*16*), and further coincidental with those reached by Yasuda (*20*) and Randall (*21*) in their study of the problem in human brain.

Table VI shows that, determined on the basis of " essential lipid," cholesterol and cerebroside are in greater concentration in the white than in the grey matter, confirming the observation of Johnson, McNabb and Rossiter (*6*) in this respect.

Finally, other points of significance to be noted in these tables may be as follows:

1) Total phospholipid exists in higher concentration in the spinal cord than in any other part of the central nervous system in the ox, rabbit and hen.

2) The three phospholipids exist in the same relative concentration in the whole brain in lower vertebrates and in the cerebrum in mammals, namely, cephalin, lecithin and sphingomyelin exist in declining order of concentration in either tissue.

SUMMARY

1. The amount of cholesterol, cerebroside, total phospholipid and the constituents (*i.e.* lecithin, cephalin and sphingomyelin) contained in the four parts of the central nervous system (*i.e.* cerebrum, cerebellum, brain stem, and spinal cord) was determined in the ox, rabbit and hen and that contained in the whole brain in the snake, frog and mackerel.

2. The ox cerebrum was separated into the white and the grey matter for the analysis of the above-named substances contained in each part.

3. In the rabbit and hen, the spinal cord was found to contain cholesterol, cerebroside and total phospholipid in higher concentration than other parts of their brain.

4. Cephalin occupies the largest proportion of total phospholipid in any species of animals and in any part of the brain studied.

5. In the ox brain various lipids were contained in greater concentration in the white matter than in the grey, but the difference of lecithin content between them is not so remarkable than that of other lipids.

6. The relative concentration of cholesterol and cerebroside, as determined on the basis of " essential lipid," was greater in the white matter than in the grey, but quite the contrary was the case with that of phospholipids.

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REFERENCES

(1) Fränkel, S., and Linnert, K., *Biochem. Z.*, **26**, 44 (1910)

(2) Koch, K., and Koch, M. L., *J. Biol. Chem.*, **31**, 395 (1917)
(3) Kaucher, M., Garbraith, H., Button, V., and William, H. H., *Arch. Biochem.*, **3**, 202 (1943)
(4) Erickson, B. N., Avrin, I., Teague, D. M., and William, H. H., *J. Biol. Chem.*, **135**, 671 (1940)
(5) William, H. H., Garbraith, H., Kaucher, M., Moyer, E. Z., Allen, J. R., and Macy, I. G., *J. Biol. Chem.* **161**, 475 (1945)
(6) Johnson, S. C., McNabb, A. R., and Rossiter, R. J., *Biochem. J.*, **43**, 573 (1948)
(7) Rin, K., *Nippon Seikagakukai Si*, **18**, 87 (1944)
(8) Fukuyama, B., *Igaku Kenkyu*, **20**, 59 (1950)
(9) Mihara, T., *Kyushu Memoirs of Med. Science*, **3**, 11 (1952)
(10) Bloor, W. R., *J. Biol. Chem.*, **82**, 273 (1929)
(11) Brand, F. C., and Sperry, W. M., *J. Biol. Chem.*, **14**, 545 (1941)
(12) Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, **114**, 583 (1936)
(13) Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, **106**, 745 (1934)
(14) Sobel, A. E., and Mayer, A. M., *J. Biol. Chem.*, **157**, 255 (1945)
(15) Hack, M. H., *J. Biol. Chem.*, **169**, 137 (1947)
(16) Schmidt, G., Benotti, J., Hershman, B., and Thannhauser, S. J., *J. Biol. Chem.*, **166**, 505 (1946)
(17) Furukawa, B., Nosue, G., and Yoshikawa, H., *Med. and Biol.*, **23**, 3011 (1952)
(18) Barton, C. J., *Anal. Chem.*, **20**, 1068 (1948)
(19) Glick, I., *J. Biol. Chem.*, **156**, 643 (1944)
(20) Yasuda, M., *J. Biochem.* (Japan), **26**, 203 (1937)
(21) Randall, L. O., *J. Biol. Chem.*, **124**, 481 (1938)

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CHARACTERIZATION OF A SUCRASE IN TAKADIASTASE

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Takadiastase acts on sucrose, raffinose, and inulin, liberating fructose residues. However, whether two or more different enzymes are concerned, or a single enzyme of group-specificity is at work, has not been made clear. Especially, the question has been discussed for a long time whether inulin is attacked specifically by so-called inulase or not. The present investigation was undertaken to dissolve this problem, taking transfructosidation into consideration.

The results presented here lead to the conclusion that there is a single transfructosidase in Takadiastase, which acts on sucrose, raffinose, and inulin, and that inulase acting specifically on inulin is not present.

To establish the identity of one enzyme with another in relatively crude extracts, usually, the ratio of the enzyme activities on different substrates is determined, and examined whether or not this ratio varies on various treatments. If a single enzyme is concerned, the ratio will be always the same, no matter how the enzyme preparation may be prepared or purified. If two or more enzymes are concerned, the ratio will vary from case to case. To represent the enzyme activity, the maximum reaction velocity which is observed in the presence of a large excess of substrate has been used frequently. For this purpose, it is desirable that the maximum velocity is measured in the range of initial velocity. A similar method was adopted in the present investigation. It was, however, practically impossible to measure the maximum velocity in the present study. Hence, the initial reaction velocity at a substrate concentration of 10 per cent (*w./v.*) was used instead of the maximum velocity.

The hydrolysis of sucrose by Takadiastase has been considered to be due to glucosaccharase, but it is now elucidated that the enzyme concerned is a transfructosidase capable of transferring fructose residues from certain donors to suitable acceptors (*1*). Therefore, it is impossible to determine the true enzyme activity by measuring reducing sugars as before. When sucrose is used as substrate, not only glucose and fructose, but also a trisaccharide (consisting of one molecule of glucose and two of fructose), a tetrasaccharide (consisting of one molecule of glucose and three of fructose), and so on, are produced by the transfer of fructose residues from sucrose molecules to other sucrose molecules and further to the trisaccharide molecules formed. Water, sucrose, and all other sugars formed act as acceptors for fructose residues; moreover, sucrose and other oligosaccharides containing fructose act also as donors of fructose residues. Thus, the path of the transfer of fructose residues is very complicated, and it seems impossible to estimate all these transfers. However, only two reactions described below occur at the start, because there are only sucrose as a donor, and, water and sucrose as acceptors.

$$\text{Sucrose} + \text{water} \rightarrow \text{glucose} + \text{fructose} \quad (1)$$
$$\text{Sucrose} + \text{sucrose} \rightarrow \text{glucose} + \text{trisaccharide} \quad (2)$$

Accordingly, the sum of fructose residues transferred in these two reactions (the sum of free fructose and one-half of the fructose in the trisaccharide formed) in the range of initial velocity may be used for the estimation of the enzyme activity.

When raffinose is used as substrate, a similar method can also be employed for the estimation of the enzyme activity, assuming that only following two reactions may occur at the start.

$$\text{Raffinose} + \text{water} \rightarrow \text{melibiose} + \text{fructose} \quad (3)$$
$$\text{Raffinose} + \text{raffinose} \rightarrow \text{melibiose} + \text{tetrasaccharide} \quad (4)$$

Melibiase activity in Takadiastase was so low that it did not interfere with the study of the action of the transfructosidase on raffinose.

When inulin was used as substrate, paper chromatographic analyses showed that only free fructose was produced from inulin in the early stages of the reaction. Therefore, only the amount of fructose residues transferred to water (*i.e.*, free fructose) may be taken into consideration for the estimation of the enzyme activity.

MATERIALS AND METHODS

Enzyme Solution—" Takadiastase Sankyo " was dissolved in water, the suspension was centrifuged, and the supernatant liquid was dialyzed for 3 days in the prensece of toluence against running water. The final concentration of the solution was about 4 per cent.

Substrates—Commercial sucrose, raffinose, and inulin were used, all of which were found to be chromatographically pure.

Incubation Procedure—Mixtures of substrate, buffer (KH_2PO_4-Na_2HPO_4, pH 6.3, final concentration 0.02 *M*), and the enzyme were incubated at 35°. After incubation the reaction mixtures were heated for inactivation for 4 minutes in a boiling water-bath, and preserved with the addition of $HgCl_2$ solution to a final concentration of 0.001–0.002 *M*.

Paper Chromatography and Quantitative Estimation—The chromatograms were developed by the descending technique on filter paper (Toyo-Roshi No. 52, 2×40 cm.) with a butanol-pyridine-water mixture (6:4:3), the solvent being allowed to travel about 30 cm. (*2*). Resorcinol-HCl or benzidine-trichloroacetic acid reagent (*3*) was used to detect sugar spots. Effective separation of the sopts on all chromatograms was obtained at 25–30°, but not below 20°. Rf values at 25–30° were as follows : fructose 0.44, glucose 0.38, sucrose 0.34, trisaccharide formed from sucrose 0.26, raffinose 0.17, tetrasaccharide formed from raffinose 0.12, inilin 0.0.

Quantitative chromatograms were run usually with a batch of eleven sheets of paper in a tank, one sheet of which was sprayed with the detecting reagent for locating each sugar. From the other paper strips the areas corresponding to each sugar were cut off, and the sugars thus separated were extracted by heating the paper with a known volume of water in stoppered tubes at 70–80° for 30 minutes. The fructose contents of the extracts were determined by the colorimetric method (Roe) (*4*), by which both free and combined forms of fructose could be estimated.

On the other hand, total reducing sugars were determined directly in incubation mixtures by the titration method (Somogyi) (*5*).

RESULTS AND DISCUSSION

Range of Initial Velocity

The following experiments were made to determine the range of initial velocity :

Enzyme solutions (1 ml.) were added to each of buffered sucrose, raffinose, and inulin solutions (4 ml.), giving the final concentration of 10 per cent (*w./v.*) substrate. The mixtures were incubated for given times and then analyzed. The results are shown in Tables I, II, and III.

According to these results, the reaction velocities of sucrose, raffinose and inulin were almost constant within an incubation time of 10, 60, and 120 minutes, respectively, and hence may be considered to be initial velocities. All subsequent experiments were carried out in the range of initial velocity.

Effect of Substrate Concentration of Reaction Velocity

The following experiments were carried out to determine the sub-

TABLE I

Action on Sucrose

	Time of incubation (*min.*)				
	5	10	15	22.5	45
Fructose transferred to water (*mg.*) (A)	0.8	1.6	2.4	4.7	11.8
Fructose transferred to sucrose (*mg.*) (B)	3.1	6.6	8.7	9.7	16.8
Total transferred fructose (*mg.*) (A+B)	3.9	8.2	11.1	14.4	28.6
Ratio A/(A+B)	0.21	0.20	0.22	0.32	0.41

TABLE II

Action on Raffinose

	Time of incubation (*min.*)		
	30	60	90
Fructose transferred to water (*mg.*) (A)	6.1	11.1	15.8
Fructose transferred to raffinose (*mg.*) (B)	1.5	4.6	5.7
Total transferred fructose (*mg.*) (A+B)	7.6	15.7	21.5
Ratio A/(A+B)	0.80	0.71	0.74

TABLE III

Action on Inulin

	Time of incubation (*min.*)		
	60	120	180
Fructose transferred to water (*mg.*)	1.1	2.3	3.0

strate concentration enough to give the maximum velocity.

Enzyme solutions (1 ml.) were added to each of a series of buffered substrate solutions (4 ml.), giving the final concentrations of 1.0–12.5 per cent (*w./v.*) of the substrate. The reaction mixtures containing sucrose were incubated for 5 minutes, those containing raffinose for 60 minutes, and those containing inulin for 120 minutes. The results are shown in Figs. 1, 2, and 3.

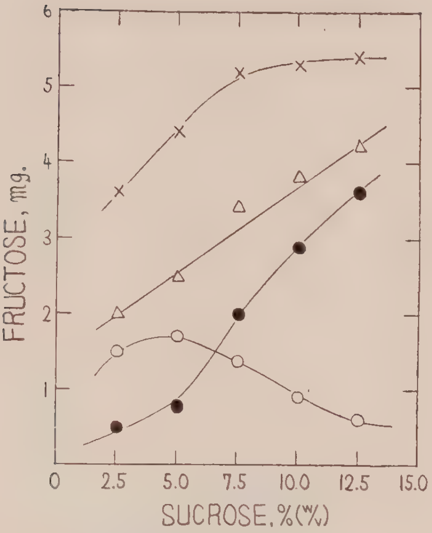

FIG. 1. Effect of sucrose concentration
○—○ Fructose transferred to water (Reaction 1) ●—● Fructose transferred to sucrose (Reaction 2) △—△ Total transferred fructose ×—× Reducing sugars as fructose.

When sucrose was used as substrate, as shown in Fig. 1, the velocity of Reaction 2 increased markedly with increasing sucrose concentration, while that of Reaction 1 reached a maximum value and then decreased. Consequently, the total reaction velocity (the sum of both reaction velocities) increased almost linearly with increasing sucrose concentration, and did not show any tendency to reach a limiting value. According to Michaelis's theory, the velocity of Reaction 1 may reach a limiting value, but the reason why the velocity decreases again, is not clear. The velocity of Reaction 2 may be expected to increase with increasing sucrose concentration, because, the higher the concentration of the acceptor (sucrose itself), the larger is the amount of fructose transferred to the acceptor. It is interesting that the rate of formation of reducing sugars showed a tendency to reach a limiting value, but the reason is not clear.

When raffinose was used as substrate, as shown in Fig. 2, the velocity of Reaction 4 continued to increase with raffinose concentration, while that of Reaction 3 approached a limiting value in accordance with

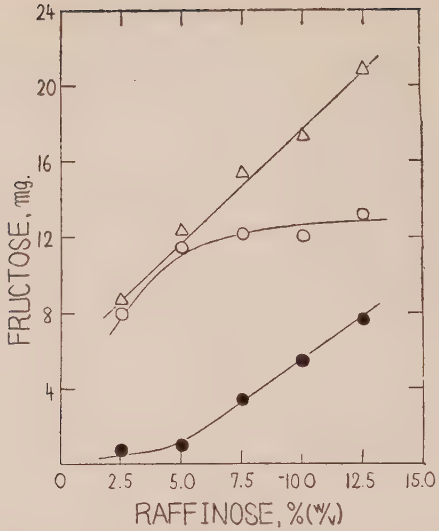

FIG. 2. Effect of raffinose concentration.
○—○ Fructose transferred to water (Reaction 3) ●—● Fructose transferred to raffinose (Reaction 4) △—△ Total transferred fructose.

Michaelis's theory. Consequently, the total reaction velocity increased almost linearly with increasing raffinose concentration, and did not show any tendency to reach a limiting value. On the whole, the relation between reaction velocity and substrate concentration in the case of raffinose is similar to that in the case of sucrose.

When inulin was used as substrate, as shown in Fig. 3, the rate of formation of free fructose increased almost linearly with increasing inulin concentration. This is probably due to the fact that the molecular weight of inulin is extremely large and hence its molar concentration is very low. It may be possible that the velocity will reach a limiting value in the presence of a large excess of inulin, but it was difficult to make experiments at the concentration higher than 10 per cent (*w./v.*) because of its low solubility.

Regarding the total reaction velocity, after all, the maximum velocity was not observed in these experiments. However, it is expected that the initial reaction velocity is proportional to the enzyme concentration at the high concentration of substrate. The initial re-

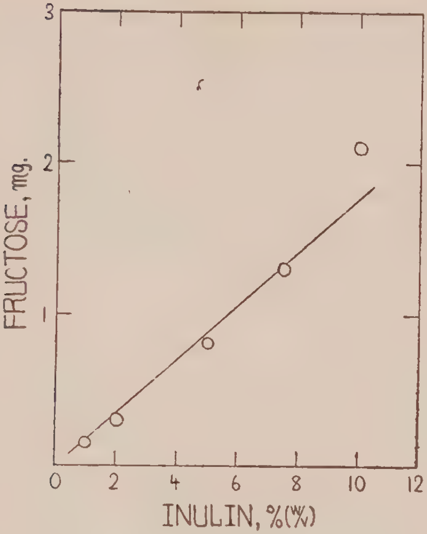

FIG. 3. Effect of inulin concentration.
○—○ Fructose transferred to water.

action velocity measured at such a concentration of substrate may be used instead of the maximum velocity to represent the enzyme activity, because the ratio of the reaction velocities on different substrates will remain constant, even when the enzyme concentration varies somewhat. It was found actually that the initial reaction velocity was proportional to the enzyme concentration at the substrate concentration of 10 per cent (*w./v.*) in all cases, and hence the enzyme activity was determined at this substrate concentration.

Ratio of the Activities on Different Substrates

The ratios of the activities of Takadiastase which had been subjected to various treatments on sucrose, raffinose, and inulin were determined at a substrate concentration of 10 per cent (*w./v.*), and compared each other, the activity on sucrose being taken for 100 (Table IV). The concentrations of all enzyme solutions were adjusted so as to give reaction velocities of about the same magnitude. Takadiastase was subjected to the following treatments:

(i) Takadiastase was dissolved in water and dialyzed. Its final concentration was about 4 per cent.

(ii) A dialyzed solution of Takadiastase was heated at 55°for 10 minutes. The activity on sucrose was reduced to about one-third by this treatment.

(iii) Takadiastase (5 g.) was dissolved in water (50 ml.) and centrifuged. The volume of the supernatant was made to 60 ml. by adding 1/5 *M* acetate buffer (pH 4.8). Methanol (60 ml.) was added, and the precipitate formed was removed. Methanol (36 ml.) was added further to the supernatant, and the precipitate formed again was collected and dissolved in water ; the solution was used as an enzyme solution after dialysis.

(iv) Takadiastase (6 g.) was dissolved in water (50 ml.) and centrifuged. Two volumes of saturated $(NH_4)_2SO_4$ solution were added to the supernatant. The precipitate formed was collected and dissolved in water ; the solution was used as an enzyme solution after dialysis.

(v) Takadiastase (5 g.) was dissolved in water (30 ml.) and centrifuged. Japanese acid clay (5 g.) was added to the supernatant adjusted to pH 5 by acetic acid, and the suspension was stirred for a while. From the clay collected and washed once with water, the enzyme was eluted with 30 ml. of 0.2 per cent $NaHCO_3$ solution ; the eluate was used as an enzyme solution after dialysis.

TABLE IV

Ratio of the Activities

	Substrate		
Enzyme soln.	Sucrose	Raffinose	Inulin
I	100	31	1.9
II	100	36	2.1
III	100	30	1.4
IV	100	34	2.1
V	100	28	1.4

As shown in Table IV, the ratio of the activities on sucross, raffinose, and inulin remained almost constant even when Takadiastase was subjected to various treatments. Furthermore, the ratio, (fructose transferred to water) / (total transferred fructose), fluctuated only in a narrow range (0.19–0.39 for sucrose, 0.68–0.91 for raffinose) throughout the experiments. These results lead to the conclusion that the catalyst in Takadiastase attacking sucrose, raffinose, and inulin is a single transfructosidase, which transfers fructose residues from the substrates not only to water, but also to the substrates themselves. There is little reason to presume the presence of inulase in Takadiastase which acts on inulin specifically. Even if inulase should be present, its activity would be low compared with that of the transfructosidase.

SUMMARY

For the characterization of a sucrase in Takadiastase, the ratio of the activities on sucrose, raffinose, and inulin was determined at the substrate concentration of 10 per cent (*w./v.*). The ratio of the activities on these three substrates remained almost constant even when Takadiastase was subjected to various treatments. The results suggested that there was a single transfructosidase attacking these three substrates in Takadiastase.

The author is very grateful to Prof. Shin'ichi Shibuya for his kind encouragement and advice. This work was aided by the Scientific Research Funds from the Ministry of Education, to which the author gave thanks.

REFERENCES

(*1*) Bealing, F. J., and Bacon, J. S. D., *Biochem. J.*, **53**, 277 (1953); Bealing, F. J., *Biochem., J.*, **55**, 93 (1953) ; Bacon, J. S. D., and Bell D. J., *J. Chem. Soc.*, 2528 (1953).
(*2*) Jeanes, A., Wise, C. S., and Dimler, R. J., *Anal. Chem.*, **23**, 415 (1951)
(*3*) Bacon, J. S. D., and Edelman, J., *Biochem. J.*, **48**, 114 (1951)
(*4*) Roe, J. H., *J. Biol. Chem.*, **107**, 15 (1934)
(*5*) Somogyi, M., *J. Biol. Chem.*, **117**, 771 (1937)

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ON THE FORMATION OF GENTISIC ACID FROM HOMOGENTISIC ACID BY THE LIVER EXTRACT*

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Homogentisic acid is oxidatively cleaved by homogentisicase to fumarylacetoacetic acid which is subsequently hydrolyzed to fumaric acid and acetoacetic acid (*1*). According to Suda and Takeda (*2*) the activity of homogentisicase is inhibited by $\alpha\alpha'$-dipyridyl and restored by the addition of ferrous ion.

In the course of our studies on the relationship between the enzymic decomposition of homogentisic acid and the metal catchers, the authors have found that gentisic acid is formed from homogentisic acid by liver extract, using nitroso-R salt as the metal catcher.

EXPERIMENTAL

Materials

Homogentisic Acid—Isolated from the urine of a alcaptonuria patient (m.p. 146°)

Nitroso-R Salt—Preparation of Takeda Pharmaceutical Ind. Ltd.

αα′-Dipyridyl—Preparation of Wako Pure Chemicals Ind. Ltd.

Liver Extract (*Crude Enzyme Solution*)—The rabbit was sacrificed by bleeding and the liver ground with an equal amount of sea sand. It was extracted three times with ice cold *M*/10 phosphate buffer (pH 7.2) for one hour at 0–4° and centrifuged at 4000 r.p.m. The supernatant was used as the crude enzyme solution. All the isolation supernatant was used as the crude enzyme solution. All the isolation procedures were carried out in the cold (0–4°).

METHODS

Determination of O_2-Uptake—O_2-uptake was measured with Warburg's manometer

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[*] The compendium of this study was announced at the 28th Annual Meeting of the Japanese Biochemical Society on 2, Nov., 1955.

[*] This study was supported by a grant for scientific research from the Ministry of Education. We should like to express our hearty thanks.

and the content of each compartment was as follows :

Main Compartment : 1.0 ml. of enzyme solution, 0.2 ml. of 1 *M* phosphate buffer (pH 7.2) and 0.3 ml. of *M*/100 *aa'*-dipyridyl or nitroso-R salt ; the mixture was made up to 2.5 ml. by addition of distilled water.

Side Arm : 0.5 ml. of homogenisic acid (2 μM).

Center Well : 0.1 ml. of 50 per cent KOH.

After the solutions were incubated at 37.5° and the manometer stand came to constant, the enzyme and substrate were mixed and the vessel was shaken.

Determination of Acetoacetic Acid—Acetoacetic acid was determined by the aniline citrate method of Edson (*3*).

Paperchromatography—After the reaction ceased the solution was acidified with H_2SO_4 and filtered and the filtrate was extracted with two times the volume of ether. The ether extract was evaporated to one third of the original volume and applied to paperchromatography. The developing solvent was a mixture of butanol, acetic acid and water (4:1:5).

RESULTS

Decomposition of Homogentisic Acid and O_2-Uptake—In the absence of the metal catcher, the liver extract decomposed the homogentisic acid completely within 10 minutes with uptake of about two atoms of oxygen per one molecule of homogentisic acid and the theoretical amount of acetoacetic acid was found. When nitroso-R salt (final concentration, *M*/1000) was added to the extract, the uptake of oxygen increased very slowly and reached the maximum after two hours. In this case 2–2.5 atoms of oxygens were consumed per one molecule of substrate but acetoacetic acid was formed in a yield of only 10–20 per cent of the theoretical value calculated from the O_2-uptake. Neither O_2-uptake nor acetoacetic acid was found by the addition of *αα'*-dipyridyl and homogentisic acid was recovered. By addition of both the metal catchers, the O_2-uptake reached the maximum more rapidly than by individual addition, consuming 2–2.5 atoms of oxygen per one molecule of homogentisic acid. Acetoacetic acid, however, was never detected in this case. (Table I and Fig. 1).

Papercharomatography—Only the presence of the three addenda, enzyme, substrate and nitroso-R salt, in the reaction solution (No. 3 and No. 4 of Table I) gave three fluorescent spots with Rf values of 0.51, 0.79 and 0.88. The spot of Rf 0.88 was bluish and most distinct of the three and the other two were yellowish. The former reduced ammoniacal alkaline $AgNO_3$ and gave a positive $FeCl_3$-reaction (yellow) and Pauly's diazoreaction (red) but the latter two did not (Table II).

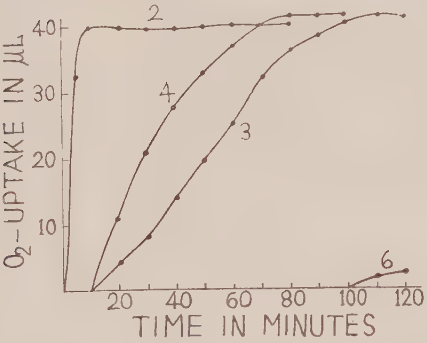
TABLE I

Decomposition of Homogentisic Acid

No.	Addition				O_2-uptake	Formation of acetoacetic acid	Blue fluorescent spot
	HGA	E	NTR	DPL			
					(μM)	(μM)	
1	+	−	−	−	0	0	−
2	+	+	−	−	1.75	0.85	−
3	+	+	+	−	1.88	0.15	+
4	+	+	+	+	1.82	0	+
5	+	+	−	+	0	0	−
6	+	−	+	−	0	0	−
7	+	E*	−	−	0	0	−
8	+	E*	+	−	0	0	−

HGA : Homogentisic Acid, E : Enzyme solution
NTR : Nitroso-R Salt E* : Enzyme solution heated at
DPL : $\alpha\alpha'$-Dipyridyl 100° for 5 minutes.

FIG. 1. Oxygen Uptake under Various Conditions.



The curve numbers represent the same conditions as shown in Table I.

Isolation and Identification of Gentisic Acid—For the isolation of the substance which gave the spot of Rf 0.88, the following experiments were conducted.

TABLE II

Properties of Each Spot

Spot	Rf	Fluorescence	$AgNO_3$	Fe	Millon	Pauly
a	0.88	bluish	+	yellow	−	+
b	0.79	yellow	−	−	−	−
c	0.51	yellow	−	−	−	−

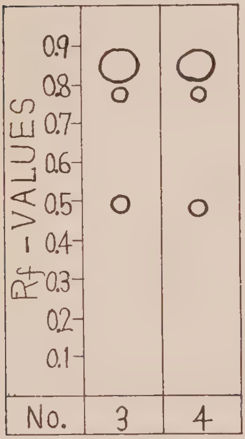


FIG. 2. Paper chromatograms of reaction mixture.

Each No. represents conditions at Table I.

Enzyme solution	100 ml.
1 *M* Phosphate buffer (pH 7.2)	20 ml.
M/100 Nitroso-R Salt	90 ml.
Homogentisic acid (90 mg.)	10 ml.
Distilled water	80 ml.
Total	300 ml.

The solution was adjusted to pH 7.2 and incubated at 37.5° for 2.5 hours under shaking. After the reaction, the solution was treated as shown in the next scheme, and yellowish, needle-like crystals were obtained. Recrystalization from the mixture of acetone and ligroin gave colourless crystals. From 2.0 g. of homogentisic acid 20 mg. of pure crystal was obtained.

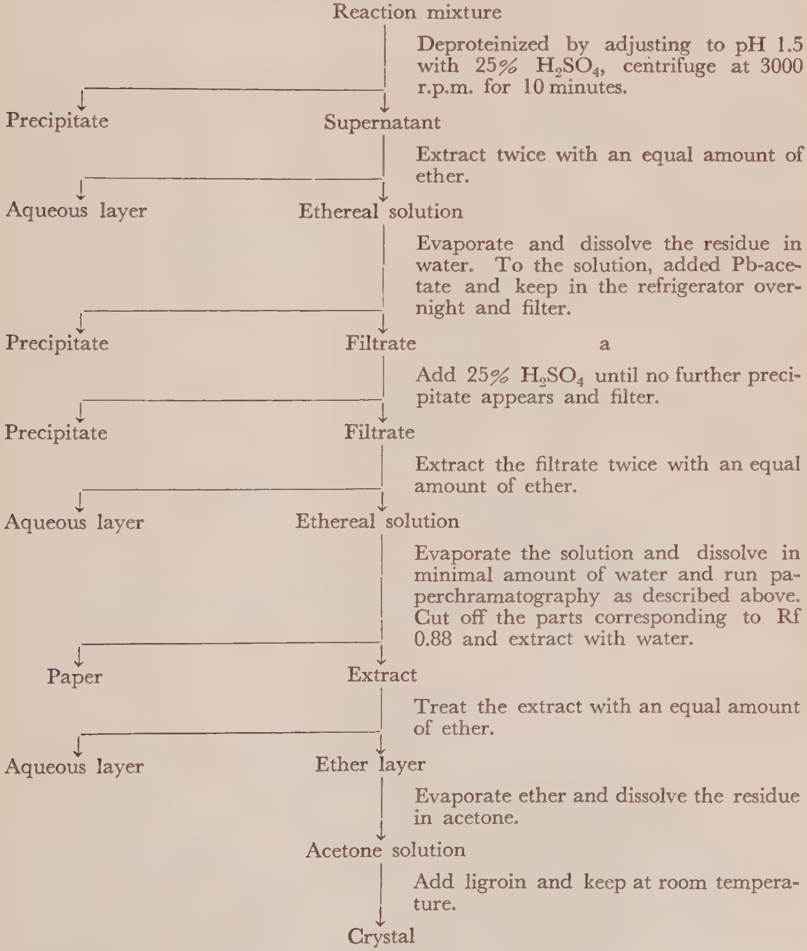
Properties of the crystal: It was readily soluble in water, alcohol, ether and acetone but insoluble in petroleum ether, benzol, ligroin and chloroform. The aqueous solution gave a violet blue color by addition of $FeCl_3$ and rapidly turned yellow. It gave the Brigg's and Pauly's diazo-reaction. The melting point was 197–201°.

These properties and the analytical data coincided with that of gentisic acid. Mixture with a synthetic specimen showed no depression in the melting point. From these results the crystal was identified as gentisic acid.

Gentisic acid in the Urine—200 mg. of nitroso-R salt was injected intraperitoneally in a male rabbit, 1.5 kg. in weight, and at the same time 2.0 gm. of L-tyrosine per kg. of body weight was administered with the stomach tube. The urine was collected in a vessel, in which diluted

	C	H	O	Mol. wt.
Sample	54.26%	4.00%	41.74%	145
$C_7H_6O_4$	54.55	3.92	41.53	154

Scheme of Isolation of Gentisic Acid



Reaction mixture

Deproteinized by adjusting to pH 1.5 with 25% H_2SO_4, centrifuge at 3000 r.p.m. for 10 minutes.

Precipitate

Supernatant

Extract twice with an equal amount of ether.

Aqueous layer

Ethereal solution

Evaporate and dissolve the residue in water. To the solution, added Pb-acetate and keep in the refrigerator overnight and filter.

Precipitate

Filtrate a

Add 25% H_2SO_4 until no further precipitate appears and filter.

Precipitate

Filtrate

Extract the filtrate twice with an equal amount of ether.

Aqueous layer

Ethereal solution

Evaporate the solution and dissolve in minimal amount of water and run paperchramatography as described above. Cut off the parts corresponding to Rf 0.88 and extract with water.

Paper

Extract

Treat the extract with an equal amount of ether.

Aqueous layer

Ether layer

Evaporate ether and dissolve the residue in acetone.

Acetone solution

Add ligroin and keep at room temperature.

Crystal

H_2SO_4 was previously placed. The 24 hours-urine was extracted with an equal amount of ether. The ether extract was evaporated and the residue was dissolved in a minimal amount of water. The solution was used for papercharomatography as described above. Three fluorescent spots appeared, Rf 0.90, 0.86–0.88 and 0.80 respectively. The spot of Rf 0.80 coincided with that of homogentisic acid and the spot of Rf 0.86–0.88 with that of gentisic acid. The urine of the tyrosine administrated control animal did not give such spots. (Table III).

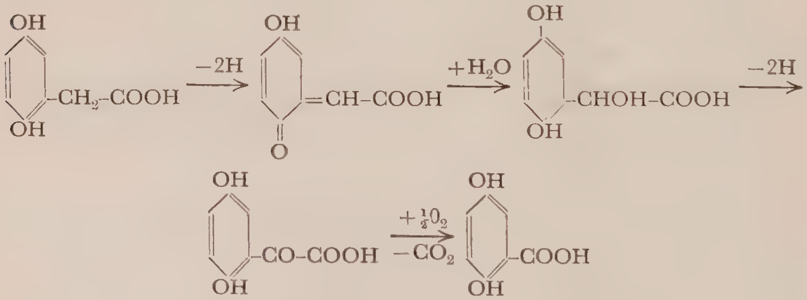
TABLE III

Properties of Each Spot

Spot	Rf	Fluorescence	$AgNO_3+NH_4OH$	Millon	Pauly
a	0.90	—	+	red	orange yellow
b	0.86–0.88	bluish	+	—	red
c	0.80	—	+	yellow	violet brown

DISCUSSION

From the above results, homogentisic acid was found to be converted to gentisic acid by a pathway differing from that known previously. Abbreviation of the side chain may be presumed according to the following scheme.



The formation of CO_2 was proven by means of Warburg's manometer. The reaction mechanism, properties of the enzymes, the presence of dehydrogenase and isolation of the intermediary metabolites are all problems to be studied in the future. From the results obtained with other metal catchers, it is suggested that the action of nitroso-R salt can be attributed to the trapping of metal. The conversion of homogentisic acid to gentisic acid was also proven by the authors using oxine (8-hydroxyquinoline) and Mitsuhashi (*4*) identified a feeble fluorescent spot correscponding to gentisic acid as well as two other spots of stronger yellow fluorescence using *o*-phenanthroline. The yellow spots corresponded to that of b and c in the Table II. The two spots may be expected to be intermediary metabolites between homogentisic acid and gentisic acid. Xanthurenic acid (similar in structure to oxine) is also an interesting substance in this respect.

Recently Arima (*5*) reported that gentisic acid was decomposed oxidatively by gentisicase isolated from bacteria, and that this too was activated by ferrous ion. Accordingly it can be assumed that the normal pathway of homogentisic acid was altered by nitroso-R salt and gentisic acid is formed. The decompostion of the latter is also inhibited by this metal catcher with accumulation of gentisic acid.

Nakamura (*6*) found gentisic acid in the urine of vitamin C deficient guinea pig administered tyrosine, and Inamori (*7*) detected this acid in the urine of alcaptonuria by means of paperchromatography. Separate determination of homogentisic acid and gentisic acid in the same urine may be possible by of enzymic decomposition of one or the other.

SAMMARY

1. Gentisic acid was isolated in crystalline form from homogentisic acid by reaction with liver extract of the rabbit in the presence of nitroso-R salt.

2. When nitroso-R salt was injected intraperitoneally in the tyrosine fed rabbit, homogentisic acid and gentisic acid were detected in the urine by means of paperchromatography.

REFERENCES

(1) Ravdin, R. G., and Crandall, D. I., *J. Biol. Chem.*, **189**, 137 (1951)
(2) Suda, M. and Takeda, Y., *J. Biochem.* **37**, 381 (1950)
(3) Edson, N. L., *Biochem. J.*, **29**, 2082 (1935) ; **29**, 2498 (1935)
(4) Mitsuhashi, T., unpublished.
(5) Arima, K., 28th Annual Meeting of Biochemical Society in Japan (1955)
(6) Nakamura, K., unpublished.
(7) Inamori, K., unpublished.

The Journal of Biochemistry, Vol. 43, No. 2, 1956

BIOCHEMICAL STUDIES ON THE FORMATION OF THE SILKPROTEIN

III. THE CONVERSION OF C^{14}-LABELED PHENYLALANINE TO TYROSINE IN THE SILKWORM LARVA (BOMBYX MORI)

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(Received for publication, January 10, 1956)

In mammals, the conversion of phenylalanine to tyrosine has been studied as part of a metabolic pathway that phenylalanine is cleaved to form acetoacetic acid via homogentisic acid (*1*, *2*). Womack and Rose (*3*) indicated that phenylalanine was converted to tyrosine in vivo by showing that tyrosine is not essential to when sufficient phenylalanine is available. Moss and Schoenheimer (*4*) demonstrated that the phenylalanine labeled with deuterium fed to rats appeared as labeled tyrosine in their body proteins. Recently, Udenfriend (*5*) demonstrated that the conversion of L-phenylalanine to tyrosine was catalyzed by an enzyme system found in mamalian livers. In insects, it has been clarified that tyrosine is further metabolized to form pigments (*6*, *7*, *8*), but little is known of the conversion of phenylalanine to tyrosine. Recently, Kirimura (*9*) concluded from a quantitative study that the tyrosine in the silkworm must be partly synthesized by this insect itself as its amount in mulberry leaves which were actually digested by the insect does not account for the higher content of this amino acid in the cocoon fibre. Yet, little is known of its precursor.

The present work was carried out, using C^{14}-labeled phenylalanine, to clarify whether a precursor of tyrosine in the silkworms is phenylalanine or not. C^{14}-labeled phenylalanine was injected into the silkworm body, and tyrosine was isolated from the cocoon fibres produced; its radioactivity was measured, and its was clarified that tyrosine is synthesized from phenylalanine in vivo.

EXPERIMENTAL

Injection of Phenylalanine-2-C^{14} into the Silkworm—L-Phenylalanine-2-C^{14}, CH_2-$C^{14}HNH_2COOH$, containing 0.84 mc per mM was obtained from the Radiochemical

Centre, Amersham, England. 0.5 μc of this phenylalanine (99 μg.* as amino acid) was injected per larva of Si 122 × Nichi 122 ♂ on the fourth day of 5th instar. Specific activity of the cocoon fibres worked out by these silkworms was measured with an SC-16 gas flow windowless counter (Tracerlab Inc.). The value observed was 4,154 cpm/100 mg. (average).

Separation of Fibroin and Sericin from Cocoon Fibres—4.86 g. of the cocoon fibres were cut to pieces, and sericin was washed out by boiling with 100 ml. of distilled water and 50 ml. of *M*/50 sodium carbonate solution repeatedly until the weight of fibroin became constant. Fibroin was washed with ethanol and ether, and dried. The yield of fibroin was 3.47 g. (71 per cent. of the cocoon fibres originally used). The combined washings containing sericin were kept frozen at −10° overnight. The sericin obtained by thawing the ice at room temperature was washed with cold water, and dried. The yield was 0.37 g. (8 per cent).

Isolation of Tyrosine, Glycine and Alanine in Fibroin—3.47 g. of fibroin were hydrolyzed with 20 ml. of concentrated hydrocholoric acid for 6 hours as usual. The hydrolysate was concentrated to a syrup under reduced pressure, and to it added, with vigorous stirring, 10 ml. of concentrated ammonia. The mixture was concentrated to remove ammonia under reduced pressure. The syrup was dissolved in hot water and the solution was decolorized with charcoal. The filtrate was cooled and placed in a refrigerator overnight. The precipitated tyrosine was recrystallized from hot water until its specific activity became constant. The yield was 194 mg. (6 per cent. of fibroin). To the filtrate from which tyrosine had been removed were added 7.5 g. of 5-nitronaphthalene-1-sulfonic acid (*10*). After the sulfonic acid was dissolved by heating, the solution was placed in the refrigerator overnight. The crystalline glycine salt was recrystallized from hot water. The yield was 4.34 g. The glycine salt was dissolved in hot water, and a saturated barium acetate solution was added. From the cooled mixture the precipitate was removed, and the filtrate was exactly freed of barium and sulfate ions by addition of 10 per cent. sulfuric acid. Acid-washed charcoal was used to aid the barium sulfate filtration. The filtrate was concentrated to dryness under reduced pressure. Glycine was recrystallized from water by adding absolute ethanol repeatedly until its specific activity became constant. The yield was 101 mg. (3 per cent. of fibroin).

To the filtrate and washings from which glycine had been removed as a salt of 5-nitronaphthalene-1-sulfonic acid were added 4 ml. of methyl cellosolve and 7 g. of azobenzene-*p*-sulfonic acid (*10*). The mixture was heated to a solution, then cooled and placed in the refrigerator overnight. The precipitated alanine salt was recrystallized from hot water and dried. The yield was 4.21 g. The liberation of alanine from alanine azobenzene sulfonate was carried out in the same manner as glycine was separated from the glycine salt. The yield of alanine was 145 mg. (4 per cent of fibroin).

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* In the injection of non-isotopic L-phenylalanine, a dose exceeding 200 μg. often resulted in deaths of larvae just before the spinning of cocoons.

RESULTS

Radioactivities of the proteins and the amino acids isolated from the cocoon fibres are summarized in Table I. The specific activity of tyrosine is strikingly high.

Identification of Tyrosine

Paper Chromatogram—The isolated tyrosine was analyzed by paper chromatography, using phenol / 0.1 per cent ammonia (85:15) as the solvent. A single spot appeared on the chromatograms, and the range of its R_F value was 0.40–0.56 which agreed with that of authentic specimen of L-tyrosine.

TABLE I

Radioactivities of the Proteins and the Amino Acids Isolated from the Cocoon Fibres

Isolated materials	Specific activity	
Sericin protein	1,407	cpm./100 mg.
Fibroin protein	4,531	"
Tyrosine	41,147	cpm./mM
Alanine	154	"
Glycine	108	"

Absorption Spectra—Absorption spectra of tyrosine in aqueous solution (0.015 per cent.) observed with Beckman Spectrophotometer agreed well with that of L-tyrosine specimen and showed a maximum peak at 275 mμ.

Ninhydrin, Millon and Diazo Reactions—All these reactions were positive.

Radioactivity of the Isolated Tyrosine—As showed in Table I, the specific activity of tyrosine obtained with the SC-16 gas flow windowless counter was strikingly high, 41,147 cpm./mM.

Radioactivity of the Chromatogram of Isolated Tyrosine—The isolated tyrosine was analyzed by paper chromatography. The strip was sprayed with ninhydrin in the usual way, the position of tyrosine marked, and the strip scanned for radioactivity by the SC-16 gas flow windowless counter (Fig. 1). As shown in Fig. 1, a comparatively high radioactivity was recognized at the position of tyrosine.

Radioautograph—A radioautograph obtained by superposition of an X ray film at 1° for two weeks on the chromatogram of tyrosine isolated gave an image at the position of tyrosine.

These results seem to suggest that isolated tyrosine is chemically pure and also labeled actually with isotopic carbon.

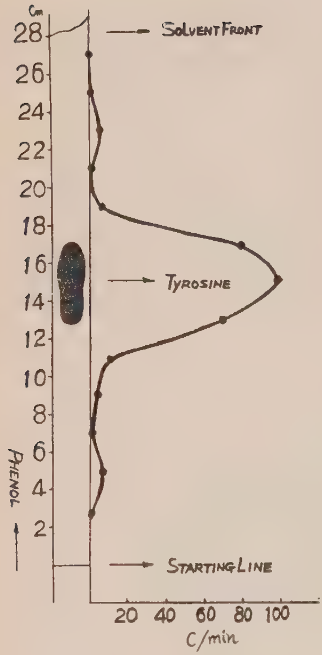

FIG. 1. Radioactivity of chromatogram of the isolated tyrosine.

DISCUSSION

Silkworms (*Bombyx mori*) eat mulberry leaves and spin their cocoons, but the content of each amino acid in the silkproteins (*11*) is strikingly different from that of mulberry leaves (*12*). Especially, the presence of tyrosine in the mulberry leaves, as it is one of the main constitutent amino acids of the silkproteins, had been viewed with the suspicious eyes. However, the presence of tyrosine in the mulberry leaves was recently ascertained by paper chromatography (*13*) and the microbiological method (*9*). In view of the high tyrosine content of the cocoon fibres, Holtzmann (*14*) compared quantitatively the tyrosine intake of the silkworms with the tyrosine content of the cocoon fibres produced, and indicated that there was such a large amount of tyrosine in the mulberry leaves, hence, no need to have recourse to the supposition of tyrosine synthesis on the part of the silkworm. Recently Kirimura (*9*) carried out the same experiment, using the microbiological method instead of the Folin and Marenzi's method adopted by Holtzmann for the assay of tyrosine, and claimed that tyrosine must be synthesized in vivo. The present work was carried out, using isotopic phenylalanine, to ascertain whether tyrosine is synthesized in the silkworm body or not, and clarified that tyrosine is synthesized from phenylalanine *in vivo*.

In the previous paper (*11*), it was indicated that some parts of tyrosine in the silkproteins were directly derived from free tyrosine present in the body fluid of the silkworm. From the present work, it is evident that the tyrosine is synthesized from phenylalanine in the silkworm body and then is utilized for the biosynthesis of the silkproteins.

In insects including the silkworm, tyrosine is known to be metabolized further, for instance, to form melanin *via* DOPA. On the other hand, there is, as shown in Table II, remarkably abundant tyrosine in the silkglands of the matured silkworms. These facts seem to suggest that some parts of tyrosine synthesized from phenylalanine are indeed further metabolized to form melanin *via* DOPA, but a larger portion of tyrosine is, as it is, used chiefly for the biosynthesis of the silkproteins, especially in the case when the biosynthesis of the silkproteins in the cells of the silkgland is remarkably active. This is peculiar when compared with the metabolism of phenylalanine in mammals and other insects.

TABLE II

The Tyrosine Content of Various Tissues at the Seventh Day of Fifth Instar

(Values in mg./worm)

Tissues	Tyrosine content*
Alimentary canal except contents	0.78
Body fluid	3.63
Silkglands including silk-substances	24.00
Silkglands except silk-substances	1.13
Fat tissues	0.69
Trachea	0.09
Malpighian tube including contents	0.03
Skin and muscle	3.00
Testis	0.04

* The determination of tyrosine in various tissues of a silkworm was carried out by the method described by Folin and Marenzi (*15*).

SUMMARY

1. To clarify the conversion of phenylalanine to tyrosine in the silkworm, isotopic phenylalanine-2-C^{14} was injected into the silkworm body at the 5th stage, and the radioactivity of tyrosine isolated from the

produced cocoon fibres was detected.

2. Tyrosine, being one of the main constituent amino acids of the silkprotein, is synthesized from phenylalanine in the silkworm body and then is used for the biosynthesis of the silkproteins.

REFERENCES

(*1*) Schepartz, B., and Gurin, S., *J. Biol. Chem.*, **180**, 663 (1949)
(*2*) Schepartz, B., *J. Biol. Chem.*, **193**, 293 (1951)
(*3*) Womack, M., and Rose, W. C., *J. Biol. Chem.*, **107**, 449 (1934)
(*4*) Moss, A. R., and Schoenheimer, R., *J. Biol. Chem.*, **135**, 415 (1940)
(*5*) Udenfriend, S., and Cooper, J. R., *J. Biol. Chem.*, **194**, 503 (1952)
(*6*) Raper, H., *Biochem. J.*, **21**, 89 (1927)
(*7*) Mason, H., *J. Biol. Chem.*, **172**, 83 (1948)
(*8*) Kikkawa, H., Ogita, Z., and Fujito, S., *Science*, **121**, 43 (1955)
(*9*) Kirimura, J., unpublished
(*10*) Carter, H. E., *Biochem. Preparations*, **1**, 15, 20 (1949)
(*11*) Fukuda, T., Kirimura, J., Matuda, M., and Suzuki, T., *J. Biochem.* **42**, 341 (1955)
(*12*) Katayama, E., *Bull. Sericul. Exp. Sta.* (*Japan*), **1**, 1 (1915)
(*13*) Fukuda, T., *Bull. Sericul. Exp. Sta.* (*Japan*), **13**, 418 (1951)
(*14*) Holtzmann, J., *Biochem. J.*, **30**, 28 (1936)
(*15*) Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 89 (1929)

The Journal of Biochemistry, Vol. 43, No. 2, 1956

STUDIES ON AMYLASE FORMATION BY BACILLUS SUBTILIS

I. EFFECT OF HIGH CONCENTRATIONS OF POLYETHYLENE GLYCOL ON AMYLASE FORMATION BY BACILLUS SUBTILIS

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(Received for publication, January 10, 1956)

It was found previously (*1*) that, when the cells of several amylase producing strains of *Bacillus subtilis* were grown aerobically in shaken cultures and then left in a fairly anaerobic condition without further aeration, a very rapid autolysis occurred. This phenomenon, called " anaerobic lysis," is not attributable to an external substance but to an endogenous factor of the cell and is pronounced only in the phase of active amylase production. When cells are cultivated in media which are suitable for good growth but not for amylase production this phenomenon is not significant. Therefore, both phenomena, amylase production and anaerobic lysis, seem to be intimately connected with each other. In other words, it seems that when cells are producing amylase, they are very unstable and liable to autolyse rapidly under some conditions.

In the course of subsequent studies (*2*) on the nature of the primary action of autolysin, which was found in the lysate after the anaerobic lysis of *Bacillus subtilis* H (*3*), it was found that the lytic effect of lysozyme on this bacterium can be almost completely prevented by conducting the lysozyme treatment of cells in a high concentration of sucrose or polyethylene glycol (PEG). In this condition only a depolymerization of the cell wall occurred and the same spherical body which had been discovered by Weibull (*4*) in *Bacillus megatherium*, *i.e.*, the protoplast, remained stable for a long time. On the other hand, it has been known empirically for a long time that the production of amylase by the *B. subtilis* group is very high when a carbon source such as starch is used at a high concentration in the medium. Considering these facts it may be supposed that when cells are producing amylase they become unstable and highly susceptible to rapid autolysis for some reason, and that the high concentration of starch (or polysaccharides produced after the hydrolysis of starch) in the medium, causing high osmotic pressure in the solution, stabilizes these cells and prolongs the period of active amylase production. Thus the activating effect of a high concentration of carbon source such as starch might be explained.

In order to test the validity of this explanation, experiments were performed in which high concentration of PEG, a non-utilizable and non-toxic substance, was used instead of starch to obtain high osmotic pressure in the medium and its effect on the production of amylase was examined. As expected, a pronounced increase in amylase production was observed when the medium was fortified with a high concentration of PEG. These results will be reported in this paper.

METHODS

B. subtilis H, which has been used throughout the previous studies, was also used in this investigation. The method of cultivation, the composition of the media, and the method for determination of amylase activity were described in previous papers (*1*, *2*, *3*). The composition of media which have not been used previously is described in the text. The rate of cell growth was followed by cultivating cells in Monod's shaking apparatus and by taking nephthelometer readings of culture tubes inserted directly into this instrument. Oxygen uptake was followed in the conventional Warburg apparatus at 30°.

PEG is a commercial product (Carbide and Carbon Chemicals Corporation, New York,) and its average molecular weight is 400.

All experiments were performed in parallel in two sets of flasks and mean values were recorded.

RESULTS

Effect of High Concentrations of Starch on Amylase Production—The synthetic basal medium was fortified with various concentrations of starch. Cells were inoculated into these media, cultivated by shaking at 30° and the formation of amylase was followed (Table I).

As is generally known, the higher the starch concentration, the greater the amylase production.

Effect of High Concentrations of PEG on the Growth and Amylase Production of B. subtilis H—With the purpose described in the introduction, the effect of high concentrations of PEG on amylase production was examined. In order to obtain clear-cut results on the effect of PEG,

TABLE I

Effect of High Concentrations of Starch on Amylase Production by B. subtilis H

Concn. of starch (%) \ Time (*hrs.*)	77	101	121	149
2	650	760	360	
4	900	1260	760	
6	1060	1410	1260	1730
8	1060	2470	2120	1650
10	2080	2840	3730	2370

Composition of the medium: Na citrate 0.04 *M*, $(NH_4)_2HPO_4$ 0.15 *M*, KCl 0.02 *M*, $MgSO_4 \cdot 7H_2O$ 0.002 *M*, $CaCl_2$ 0.001 *M* and soluble starch as indicated in the table. pH=7.2. Temp. 30°. Shaken culture. Figures in the table indicate amylase activity in units per ml. of the medium, expressed as $D^{30'}_{40^\circ}$.

the GBY medium (glucose 0.5 per cent, peptone 1 per cent, meat extract 0.5 per cent, yeast extract 0.2 per cent, NaCl 0.2 per cent, pH 7.2), which is suitable for good growth but not for amylase production, was selected as a basal medium in one set of experiments. It has been generally known that a high concentration of phosphate is favorable for amylase production. Though this effect is usually considered to be due to the maintenance of neutral pH, it might be also due to high osmotic pressure in the medium. From these considerations, GBY medium fortified with a high concentration of phosphate (0.15 *M*) was also used as a basal medium in another set of experiments. PEG was added in varying concentrations to each of the basal media. Then the growth of cells, pH and the amount of amylase produced were followed.

From the results shown in Fig. 1 (a) and (b), many interesting facts can be recognized.

(1) Although the PEG had no effect on the growth of cells in the GBY medium in concentration up to 10 per cent, and even inhibited growth at a concentration of 15 per cent (mean generation time: control, 48 minutes; with 15 per cent PEG, 72 minutes), the production of amylase was markedly increased by high concentration of PEG, the production of amylase in the 10 per cent PEG medium being about 7 times higher than in the control at the end of the cultivation.

(2) When the cells were cultivated in the GBY plus phosphate

medium, the lag period was longer than in GBY medium and amylase production was greater. Addition of 10 to 15 per cent PEG prolonged the lag period further and also inhibited the growth rate (mean generation time: control, 48 min.; with 15 per cent PEG, 96 minutes). The production of amylase, on the other hand, was markedly increased by high concentrations of PEG. Furthermore, this activation was recognized only when the PEG concentration was very high. No effect was observed in PEG concentrations up to 5 per cent.

(3) From Fig. 1 (a) it is clear that the phase of active amylase production is distinct from that of cell multiplication. In this case the

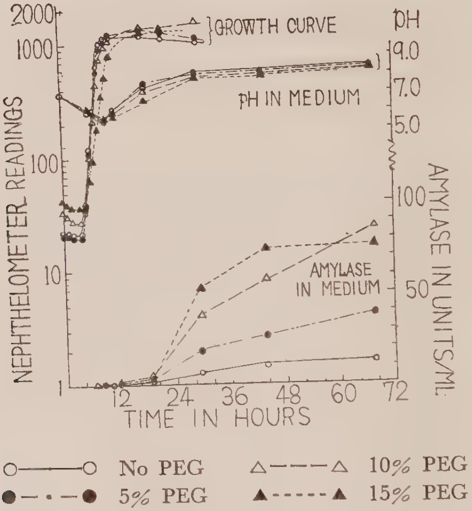

Fig. 1 (a). Effect of high concentrations of PEG on growth and amylase production by *B. subitilis* H.

Composition of the medium: glucose 0.5%, meat extract 0.5%, peptone 1%, yeast extract 0.2%, NaCl 0.2%, and varying concentrations of PEG or phosphate (0.15 *M*) plus PEG as indicated. PH 7.0.

B. subtilis H were grown in a GBY medium at 30° for 15 hrs with shaking. One ml. of a 120-fold diluted culture was transferred to 10 ml. of each of the various media described above. Temp., 30°; Shaken culture. The experiments described in figures (a) and (b) were performed simultaneously.

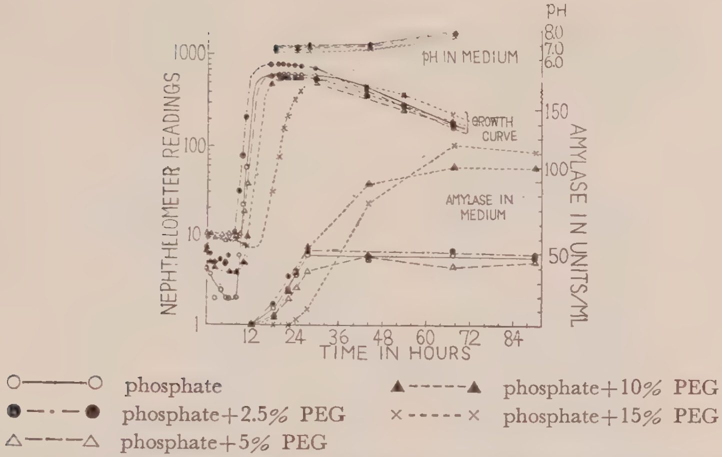

FIG. 1 (b). Effect of high concentrations of PEG on growth and amylase production by *B. subitilis* H. The conditions of experiment were the same as Fig. 1 (a)

log phase lasted from the 4th to the 8th or 9th hour, while no appreciable production of amylase could be observed until the 18th hour of cultivation. Amylase formation within the cell seems not to take place until this time either, as no amylase was detected even after the artificial lysis of cells with lysozyme. Therefore it is concluded that the amylase formation is a process different from the synthesis of normal cellular protein and takes place only in old cells. This situation can also be seen in Fig. 1 (b). In this case growth was followed for a long period until near the end of the cultivation. It can be seen that, when the cultures are shaken continuously, autolysis of the cells sets in and proceeds gradually thereafter. The production of amylase proceeds linearly in the stationary phase until the phase of actual autolysis (see curves with 0, 2.5, and 5 per cent of PEG). The addition of 10 or 15 per cent of PEG apparently prolonged the period of amylase production.

The above results were obtained in experiments where cultures were grown in Monod's shaking apparatus. An experiment similar to that described in Fig. 1 (a) was performed using the usual reciprocating shaking apparatus. In this case as well, a pronounced activation was observed in the presence of a high concentration of PEG (about 700 per cent activation in the presence of 15 per cent PEG).

Next, PEG was added to a Soy medium suitable for amylase prodution (*1*), which already contained 8 per cent of starch, 0.15 *M* phosphate and, in addition, an alkaline extract of soybeans. In this case a slight activating effect was observed when 10 or 15 per cent of PEG was used (Fig. 2).

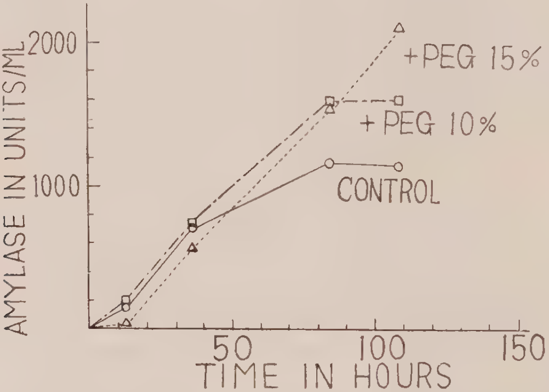

Fig. 2. Effect of high concentrations of PEG on amylase production by *B. subtilis.*

Basal medium: Soy medium (1). PEG was added to the basal medium as indicated. Temp., 30°; shaken culture.

Effect of Various Concentrations of Sucrose on the Amylase Production—It was previously recognized (*2*) that the protoplast of *B. subtilis* H is stable in sucrose as well as in PEG solutions of high concentration. However, sucrose is metabolized by this organism and consequently many complex effects may be produced, in addition to its stabilizing effect, when this sugar is present in the growth medium. Nevertheless, the effect of various concentrations of sucrose on amylase production was examined using GBY plus phsophate as a basal medium. As shown in Fig. 3, amylase production was considerably decreased in the presence of 5 or 10 per cent of sucrose. The activating effect was recognized only when the sucrose concentration was raised to 15 per cent and, even then, only in the last phase of culture.

It is probable that sucrose was metabolized and that either the decrease in pH due to acid production from sucrose (see pH curves in Fig. 3) or some other secondary changes inhibited amylase production. When the concentration of sucrose was 5 to 10 per cent this inhibitory

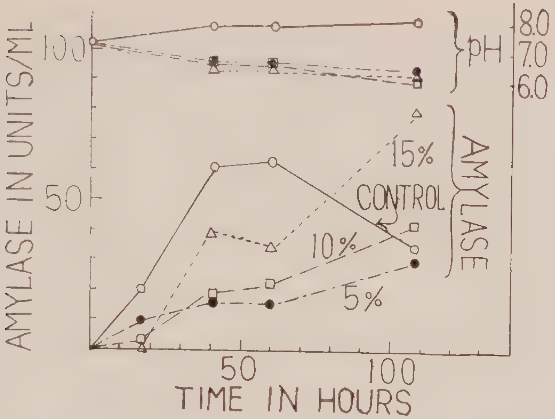

Fig. 3. Effect of various concentrations of sucrose on amylase procution by *B. subtilis* H.

Basal medium: GBY plus phosphate (0.15 *M*). Sucrose was added to the basal medium as indicated. Temp., 30°; shaken culture.

effect seems to be stronger than the activating effect; the activating effect can be observed only in a sucrose concentration of 15 per cent.

Metabolizability of PEG—The marked activating effect of PEG on amylase production seems to be due to some physicochemical effect on the medium, and not to its role as a carbon or energy source. This supposition is supported by the fact that its activating effect can be observed only when its concentration is very high (see, for example, Fig. 1 (b)). Further confirmation was made by examining the oxygen uptake of cells in the presence of this substance.

As shown in Table II, no oxidation of PEG was recognized when cells were grown either in the presence of PEG or in its absence, while glucose was oxidized rapidly in both instances. (Sometimes a slight inhibition of the autorespiration by PEG was observed. This effect seems to be due to the action of peroxide which is known to be formed spontaneously from PEG.)

Therefore, it is very likely that PEG is not a metabolizable substance and that its activating effect is of a physicochemical nature.

DISCUSSION

It was clearly demonstrated by these results that a high concen-

TABLE II

Oxidation of Glucose and PEG by B. subtilis H

Substrate	Conc. of substrate (%)	Q_{O_2}	
		(a)*	(b)*
Glucose	0.7	71.7	44.5
PEG	0.7	31.5	14.8
PEG	1.4	30.7	
Autorespiration		33.0	15.0

*(a): by the cells grown on GBY medium for 14.5 hours at 30°.
(b): by the cells grown for 20 hours at 30° on the GBY medium which contained additional phosphate (0.15 *M*) and PEG (10%).
Figures are recorded without the correction for autorespiration.

tration of PEG remarkably increased the amylase production by *B. subtilis* H, as was expected from the results of previous studies. PEG is not oxidized by this bacterium, and its effect seems to be due to a stabilization of cells during the shaking in a way analogous to that observed in the formation of protoplasts, described in a previous paper (*2*). Yet the cells grown in PEG medium with shaking are highly susceptible to anaerobic lysis in the phase of active amylase production, and protoplasts obtained from such cells are also very unstable (*5*). Therefore, amylase production and susceptibility to lysis are intimately connected in this case, too, and the concept of the instability of cells during amylase production was further strengthened. We cannot yet draw any definite conclusion as to the mechanism of activation of amylase production by PEG. It may be due to the stabilization of cells, as anticipated from the studies on protoplasts, or it may be due to some derangement of normal metabolism which causes highly unstable and abnormal physiological conditions under which the main cellular functions are forced to produce a large amount of exoenzyme. Synthesis of lysogenic bacteriophage or bacteriocine as a primary cause of this derangement, which might lead to amylase production, cannot yet be excluded ; this was discussed in the previous paper (*3*). In any event amylase production is a kind of abnormal biosynthesis distinct from normal cellular biosynthesis. In this abnormal condition the cells are highly unstable and high concentrations of suitable substances, such as PEG or starch, seem to maintain this abnormal, unstable state, thus exerting a remarkable activating effect on amylase production.

According to the recent studies of Smith *et al.* (*6*), pathogenic strains of *Bacillus anthracis*, which were obtained from a host animal, are very delicate. They are rapidly lysed in ammonium carbonate solution, while no such property is observed in organisms grown *in vitro*. *B. anthracis* is known to produce a specific extracellular lethal toxin only when grown in the host animal (*7*). Therefore, the production of this toxin and the susceptibility to lysis may have some relation.

A relationship between exotoxin production and autolysis has sometimes been reported (for example, ref. *8*), although usually it is simply stated that autolysis causes the liberation of toxin preformed within the cell into the medium.

There are many examples of abnormal biosyntheses which are lethal or pathogenic to the organism. According to Lwoff (*9*), biosynthesis of lysogenic phage or bacteriocine causes the death of a bacterium whether the synthesis is productive or not. Furthermore, the biosynthesis of protein P_z by a mutant strain of *E. coli* ML is accompanied by a very marked inhibition of its growth rate, and is regarded as a kind of disease. At present we cannot decide whether the abnormal biosynthesis of amylase is actually the cause of instability or whether it is the result of this abnormal physiological condition. Our studies on the mechanism of amylase formation are under way from these points of view.

SUMMARY

High concentrations of polyethylene glycol markedly increase amylase production by *Bacillus subtilis* H. Polyethylene glycol is not oxidized by this bacterium and its effect seems to be physicochemical, similar to its action in the formation of protoplasts, described in a previous paper.

From these, and many other experimental results obtained previously, the following explanation was proposed: Amylase production is a kind of abnormal biosynthesis occurring in the old cells in which normal cellular multiplication no longer takes place. When cells are producing amylase they are unstable and highly susceptible to rapid autolysis. They undergo rapid lysis anaerobically, and lyse gradually in shaken cultures. A high concentration of a suitable substance, such as polyethylene glycol or starch, either stabilizes these cells, or maintains this abnormal, unstable state, and prolongs the period of active amylase

production, thus displaying remarkable activating effect on the total amylase production.

REFERENCES

(*1*) Nomura, M., *J. Agr. Chem. Soc. Japan*, **29**, 674 (1955)
(*2*) Nomura, M., and Hosoda, H., *J. Bacteriol.*, in press.
(*3*) Nomura, M., *J. Agr. Chem. Soc. Japan*, **29**, 678 (1955)
(*4*) Weibull, C., *J. Bacteriol*, **66**, 688–695 (1953)
(*5*) Nomura, M., unpublished experiment
(*6*) Smith, H., Keppie, J., and Stanley, J. L., *Brit. J. Exp. Pathol.* **34**, 477 (1953)
(*7*) Smith, H., Keppie, J., Mechanisms of microbial pathogenicity, p. 126 Cambidge University Press (1955)
(*8*) Raynaud, M., *Compt. rend. Acad. Sci.*, **225**, 543 (1947); Seki, T., Takagi, M., Hirabayashi, H., and Yamaguchi, H., *Med. J. Osaka Univ.*, **5**, 271 (1954)
(*9*) Lwoff, A., The nature of virus multiplication, p. 149, Cambridge Univ. Press (1953)

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BIOCHEMICAL STUDIES ON "COENZYME SULFATE ANALOGUES"

I. EFFECTS OF ADENOSINE-MONOSULFATE AND RIBOFLAVIN-MONOSULFATE ON D-AMINO-ACID OXIDASE

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In 1953 Yamashina and one of us (Egami) found (*1*) that adenosine-monosulfate competed with DPN and thus inhibited the dehydrogenation of ethanol by yeast alcohol dehydrogenase. So we were led to consider that it would be interesting to study systematically the biochemical activities of various "coenzyme sulfate analogues," which have a sulfuric acid group in place of a phosphoric acid group of various coenzymes and related compounds. We expected, for example, that adenosine-monosulfate (AMS) and riboflavin-monosulfate (FMS) might combine with D-amino-acid oxidase in competing with FAD: the former competes probably with the adenylic acid part of FAD and the latter probably with the riboflavin phosphoric acid part of FAD, as shown schematically in Fig. 1. In this connection the present paper deals with the effects of AMS and FMS on the oxidative deamination of D-alanine by D-amino-acid oxidase.

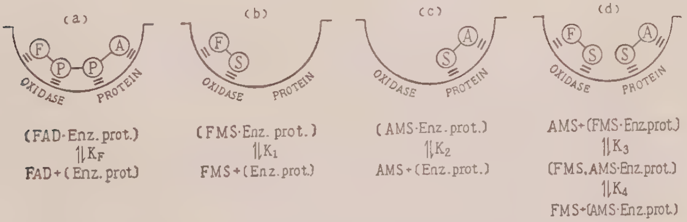

FIG. 1. Schematical representation of the possible complexes of the oxidase protein with FAD, AMS and FMS.

KINETIC CONSIDERATIONS

As is well known, the relation between D-amino-acid oxidase activity and FAD concentration is

$$v=\frac{Vf}{K_F+f} \qquad (1),$$

where v is the reaction velocity in the presence of a concentration f of FAD, V is the maximum velocity obtained by increasing the concentration of FAD, and K_F is the dissociation constant of the complex [FAD-Enzyme protein] and independent of f and v.

If an inhibitor acts by combining with the oxidase protein in competition with FAD, an equation.

$$v=\frac{Vf}{K_F\left(1+\frac{i}{K_i}\right)+f} \qquad (2)$$

may be obtained, where i is the concentration of the inhibitor, K_i is the dissociation constant of the complex [Inhibitor-Enzyme protein]. If v_o is the reaction velocity in the absence of inhibitor, from Eq. (2) we can derive the equation

$$\frac{v_o}{v}=1+\left\{1-\frac{v_o}{V}\right\}\frac{i}{K_i} \qquad (3).$$

K_i may be evaluated from Eq. (3), which gives a straight line by plotting v_o/v against i, as described by Burton (*2*).

When two inhibitors I_1 and I_2, such as AMS and FMS, are added at the same time, we must consider 3 cases:

1) FAD, I_1 and I_2 compete each other at one point of attachment on the enzyme protein. Then we may write

$$v=\frac{Vf}{K_F\left(1+\frac{i_1}{K_1}+\frac{i_2}{K_2}\right)+f} \qquad (4),$$

where i_1, i_2 are the concentrations of two inhibitors, and K_1, K_2 are the dissociation constants of the complexes [I_1-Enzyme protein] and [I_2-Enzyme protein], respectively.

2) The two inhibitors compete with FAD, but not each other as shown in Fig. 1. Then we may write in general

$$v=\frac{Vf}{K_F\left(1+\frac{i_1}{K_1}+\frac{i_2}{K_2}+\frac{i_1 i_2}{K_1 K_3}\right)+f} \qquad (5)$$

or $$v=\frac{Vf}{K_F\left(1+\frac{i_1}{K_1}+\frac{i_2}{K_2}+\frac{i_1 i_2}{K_2 K_4}\right)+f} \qquad (6),$$

where K_3 is the dissociation constant of $[I_1, I_2\text{-Enzyme protein}] \rightleftarrows I_2 + [I_1\text{-Enzyme protein}]$, K_4 is that of $[I_1, I_2\text{-Enzyme protein}] \rightleftarrows I_1 + [I_2\text{-Enzyme protein}]$. In this case $K_1K_3 = K_2K_4$.

3] If $K_3 = K_2$ and $K_4 = K_1$, *i.e.* no interaction takes place between the affinities I_1-Enzyme protein and I_2-Enzyme protein each other, then we may write

$$v=\frac{Vf}{K_F\left(1+\frac{i_1}{K_1}+\frac{i_2}{K_2}+\frac{i_1 i_2}{K_1 K_2}\right)+f} \qquad (7).$$

EXPERIMENTAL*

D-*Amino-acid Oxidase*—The protein E prepared by the method of Negelein and Brömel (*3*) was used as an enzyme preparation.

Flavin-adenine-dinucleotide (FAD)—FAD powder was prepared according to Yagi *et al.* (*4*). It was free from other flavins, nucleic acids and inorganic substances. The purity was more than 60 per cent. The dissociation constant for the enzyme protein was $1{,}1 \times 10^{-7}$ *M*.

Adenosine-monosulfate (AMS)—Sodium adenosine-5′-monosulfate synthesized by Egami and Takahashi (*5*).

Riboflavin-monosulfate (FMS)—Calcium riboflavin-monosulfate (probably 5′-sulfate) synthesized by Takahashi, Yagi and Egami. The detail of the synthesis will be published elsewhere.

D-Amino-acid oxidase activity was measured with 2 mg. enzyme preparation in Warburg manometer at pH 8.3 and 37° in the presence of 1.4×10^{-5} *M* FAD, 0.125 *M* DL-alanine, and 0.01 *M* pyrophosphate buffer in a total volume of 1.6 ml. The gas phase was air, the centre well contained 0.2 ml. 20 per cent NaOH. 10 minutes after immersing the vessel and mixing the reactants, readings were taken at 10 minutes intervals for 100～160 minutes. Any inactivation of the enzyme was not observed during the measurement. The pressure changes were

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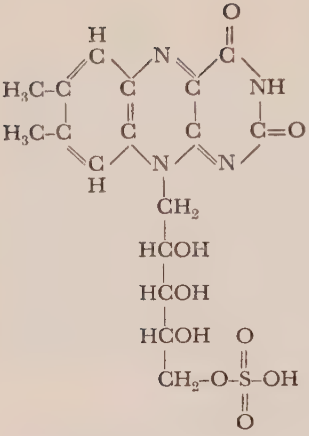
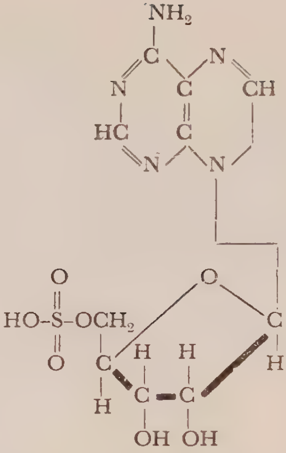
* We assumed that no direct interaction took place between AMS or FMS and FAD. The assumption is quite reasonable, because neither AMS nor FMS affected the extinction coefficient of FAD and because we obtained a straight line when plotted v_0/v against i as expected from Eq. (3), which was derived by neglecting the direct interaction of the inhibitor and FAD.



Riboflavin-5′-monosulfate



Adenosine-5′-monosulfate

plotted against time. From the slope of the straight line thus obtained the rate of O_2 uptake was calculated. The activity is expressed as μl. O_2 consumed /30 minutes. V was 66 μl. O_2/30 minutes. K_F was evaluated from the results as 1.1×10^{-7} M.

Determination of K_i—In each vessel, 2 mg. enzyme preparation, 0.125 M (final concentration) DL-alanine, 1.6×10^{-7} M (final concentration) FAD were taken in the main chamber (1.4 ml.), and $2 \times 10^{-6} \sim 4 \times 10^{-5}$ M (final concentration) FMN or $5 \times 10^{-4} \sim 2 \times 10^{-3}$ M (final concentration) AMS was taken in the side chamber (0.2 ml.) (total volume 1.6 ml.). Enzyme activity was measured for 30 minutes, then after adding the inhibitor into the main chamber, the enzyme activity in the presence of inhibitor was measured after the equilibrium had attained and expressed as μl. O_2 consumed /30 minutes. The results were summarized in Fig. 2. and Fig. 3. By several experiments, the dissociation constants of AMS and FMS for the enzyme protein were evaluated respectively as 3.12×10^{-3} M and 3.15×10^{-5} M. The former is, although a little larger, of the same order of magnitude as that obtained by Burton for adenosine-5′-phosphate (*2*). It seems to mean that the replacement of phosphoric acid group with sulfuric acid group has only a little effect on the affinity to the enzyme protein. It is remarkable that the dissociation constant of FMS for the enzyme protein is so small as 3.15×10^{-5} M. FMS may be regarded as the strongest antagonist ever known for FAD, at least concerning D-amino-acid

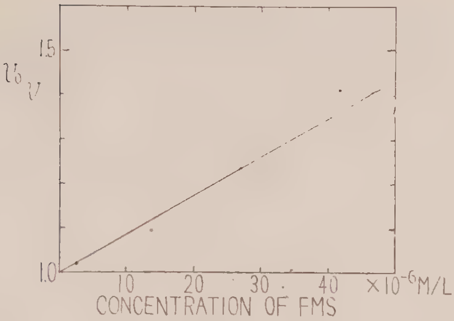


FIG. 2. Inhibition of D-amino-acid oxidase by FMS.

v_o is the reaction velocity in the absence of FMS (38,5 μl 0_2/30 min.).

v is the reaction velocity in the presence of FMS. Concentration of FAD 1,6 $\times 10^{-7}$ M.

Ki=3.15 $\times 10^{-5}$ M.

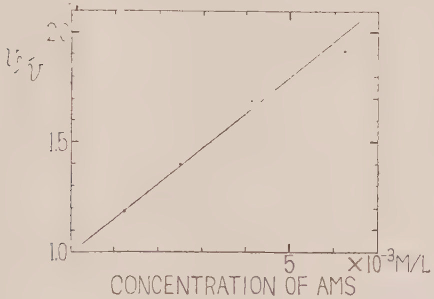


FIG. 3. Inhibition of D-amino-acid oxidase by AMS.

v_o is the reaction velocity in the absence of AMS (33,0 μl 0_2/30 min.).

v is the reaction velocity in the presence of AMS. Concentration of FAD 1.6 $\times 10^{-7}$ M.

Ki=3.12 $\times 10^{-3}$ M.

oxidase.

Enzyme Activities in the Presence of FMS and AMS—In each vessel, 2 mg. enzyme preparation, 0.125 M DL-alanine, 1.6 $\times 10^{-7}$ M FAD were taken in the chief chamber as usual, and 6.85 $\times 10^{-6}$ M FMS and 1.25 $\times 10^{-3}$ M AMS were taken in the side chamber (all expressed as final concentration in 1.6 ml.). 30 minutes after immersing the vessel in the water-bath, the reactants were mixed. The activities evaluated as μl. O_2 uptake/30 minutes are shown in Table I.

TABLE I

Enzyme Activities in the Presence of FMS and AMS

Time (*min.*)	0–30	40–70	60-90	80–110
Activities *μl. 0_2 consumed/30 min.*	32.5	29,5	27,5	27,5

The activity may be calculated theoretically from the Eq. (4) or the Eq. (7), putting the following values in the equations: $V=66$ μl./30 minutes, $f=1.59\times10^{-7}\ M$, $K_F=1.1\times10^{-7}\ M$, $K_1=3.15\times10^{-5}\ M$ (FMS), $i_1=6.85\times10^{-6}\ M$ (FMS), $K_2=3.12\times10^{-3}\ M$ (AMS), $i_2=1.25\times10^{-3}\ M$ (AMS). The calculated values from Eqs. (4) and (7) are 31.2 and 30.3 μl. O_2 uptake/30 minutes respectively. The former value is apparently too large compared with the equilibrium value (27,5 μl. O_2 uptake/30 minutes) obtained by the experiments. So it may be concluded that, as reasonably expected, AMS and FMS do not compete each other. Moreover the latter value seems to be a little too large too, exceeding the limit of experimental error. So we have been led to consider the possibility that an interaction takes place between the affinities I_1-Enzyme protein and I_2-Enzyme protein such that the affinities increase by the coexistence of the two inhibitors on an enzyme molecule. In this case we must consider the Eq. (5) or Eq. (6), and we may be able to calculate the dissociation constants K_3 and K_4 putting the value 27.5 μl. O_2 uptake/30 minutes in the equations. Thus we obtained the values, $K_4=6.7\times10^{-6}\ M$ (FMS), $K_3=6.6\times10^{-4}\ M$ (AMS). The affinities become about 5 times as large by the interaction.

SUMMARY

1. Adenosine-5′-monosulfate (AMS) and riboflavin-monosulfate (FMS) compete with FAD and inhibit the oxidative deamination of D-alanine by D-amino-acid oxidase.

2. The dissociation constants of AMS and FMS for the enzyme protein at pH 8.1 and 37° were evaluated as $3.12\times10^{-3}\ M$ and $3.15\times10^{-5}\ M$ respectively.

3. When AMS and FMS coexist, they compete with FAD, but not each other as expected and shown in Fig. 1.

4. When AMS and FMS coexist on an oxidase protein molecule, it seems an interaction to take place so that the affinities between the oxidase protein and the inhibitors may increase.

We wish to thank Miss M. Naoi, Mr. T. Ozawa and Mr. K. Okada for their kind assistance.

REFERENCES

(*1*) Yamashina, I., and Egami, F., *J. Japan. Biochem. Soc.*, **25**, 281 (1953); Egami, F., *Medicina Revuo*, **2**, 26 (1954)
(*2*) Burton, K., *Biochem. J.*, **48**, 458 (1951)
(*3*) Negelein, E., and Brömel, D., *Biochem. Z.*, **300**, 225 (1939)
(*4*) Yagi, K., Matsuoka, Y., Kuyama, S. and Tada, M., *J. Biochem.*, **43**, 93 (1956)
(*5*) Egami, F. and Takahashi, N., *Bull. Chem. Soc. Japan*, **28**, 666, (1955)

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REACTION OF VERDOHEMOGLOBIN FORMATION IN VITRO, WITH SPECIAL REFERENCE TO ITS QUANTITATIVE TREATMENT

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In our previous investigation (*1*), it was proved that the breakdown of heme in the system of hemoglobin(or myoglobin)—ascorbic acid—oxygen leading to the formation of verdohemoglobin (the immediate precursor of biliverdin) occurs in the following sequence:

Hemoglobin (or myoglobin)→choleglobin→verdohemoglobin (or verdomyoglobin)

A hypothesis for the reaction mechanism was also presented in the same work. As was then pointed out, verdohemoglobin, the immediate precursor of biliverdin, was demonstrated for the first time spectroscopically in this reaction system.

In the present report, the said reaction was quantitatively investigated mainly through the use of myoglobin as the starting material instead of hemoglobin. The reaction proceeds more smoothly in the case of myoglobin compared with that of hemoglobin, since there occurred no choleglobin-b formation in this system.

As reported by Kikuchi and Tomimura (*2*), the breakdown of hemoglobin in this reaction proceeds more rapidly when benzoate or salicylate was added to the reaction system, these reagents being effective as the " perturbator " to the protein moiety of hemoglobin. This holds also for the reaction with myoglobin and the yield in the verdohemoglobin was found to be significantly increased in this case. This seems to be of great importance to the present attempt to follow up the reaction sequence quantitatively, starting with myoglobin instead of hemoglobin.

EXPERIMENTAL

Material: Metmyoglobin was prepared in a crystalline form from equine heart by the method described by Tsushima and Okazaki (*3*). Spectrometric measurement was carried out by a photoelectric spectrophotometer Model EPB-V of Hitachi Co.

I. Effect of Benzoate upon the Reaction of Verdomyoglobin Formation

Breakdown of heme in the reaction system of myoglobin+ascorbic acid+oxygen was greatly promoted by the addition of sodium benzoate in a suitable concentration (Fig. 1). It is especially notable that the extinction at 760 mμ (specific to verdoheme) was found to be significantly higher than in the system without benzoate (cf. Fig. 3 in the

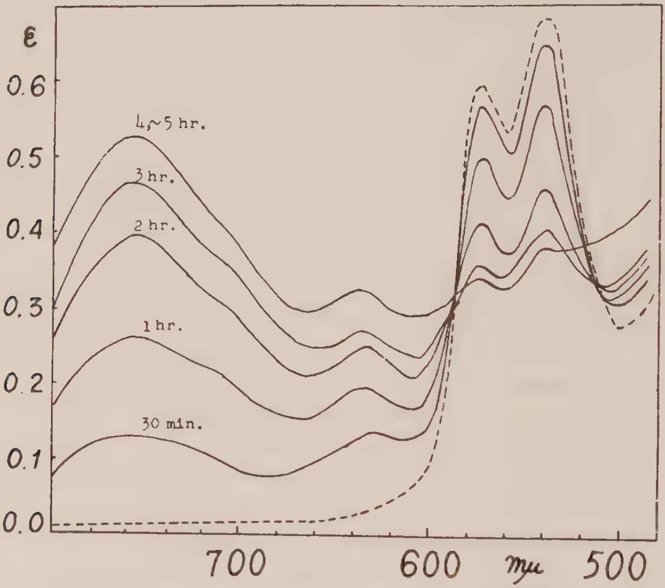

FIG. 1. Absorption change during the reaction process of verdomyoglobin formation in the presence of sodium benzoate.

Metmyoglobin, 2×10^{-4} *M*, 2.5 ml.
Ascorbic acid, 0.1 *M*, 0.5 ml.
Sodium benzoate, 5×10^{-1} *M*, 0.25 ml.
T=37°, pH 7.0

Absorption of reaction solution was measured after addition of 5 ml. 2% NaOH and a small amount of powdered $Na_2S_2O_4$, followed by saturation with CO gas.

previous communication (*1*)). This indicates that the reaction proceeds much more smoothly with the addition of benzoate.

A series of similar experiments were conducted with benzoate in varying concentrations and the absorption spectra of the reaction solutions were measured after 3 hours. Applying the equations established in the previous report to these absorption values found, the extinction of each component present in the reaction solution, namely of protoheme, choleheme, and verdoheme (x, y and z, respectively) was calculated and the values so obtained were plotted against log concentrations of benzoate added. This relationship is shown in Fig. 2. The rate of protoheme decomposition increased with the increasing concentration of benzoate added and in parallel with the increase in the rate of choleheme and verdoheme production. However, when the concentration of benzoate became excessibly higher, the protoheme decomposition became slower.

Under the same experimental conditions as shown in Fig. 2, the oxygen uptake was manometrically measured by the Warburg technique.

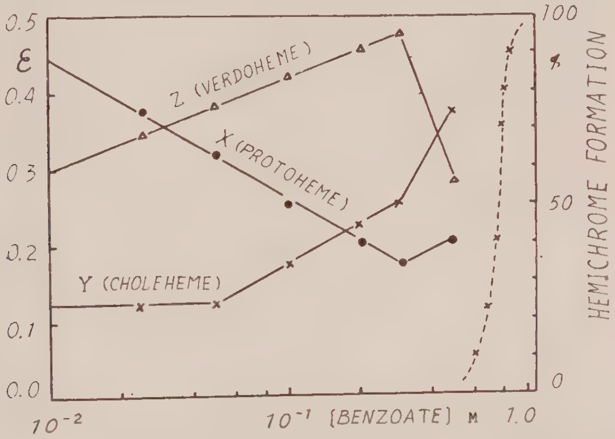

Fig. 2. Changes in the extinction of protoheme, choleheme, and verdoheme (x, y, and z, respectively) in the reaction solutions after 3 hours under the presence of benzoate in varied concentrations.

Reaction conditions and the procedure of the measurement are the same as that given in Fig. 1.

The broken line indicates the relationship between the hemichrome formation (%) from methemoglobin and the concentration of benzoate.

The rate of oxygen uptake increased with the increase of benzoate concentration (Fig. 3). At an optimal concentration range of benzoate the acceleration reached the maximum and remained constant. Above this limit concentration of benzoate, the rate of oxygen uptake again decreased. The benzoate concentration at which the oxidase activity of metmyoglobin began to decrease corresponded to that in which the spectroscopically visible degree of perturbation began to take place in the molecule of metmyoglobin as shown in Figs. 2 and 3 with broken line. The relations represented in Figs. 2 and 3 are qualitatively the same as those which Kikuchi and Tomimura have previously demonstrated in similar experiments using hemoglobin.

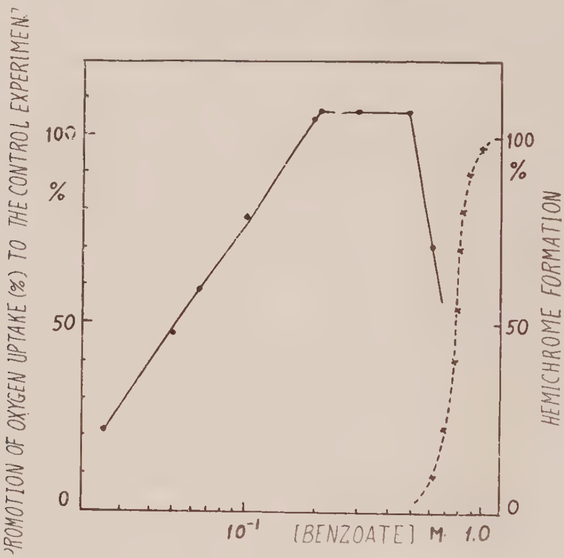

Fig. 3. Promotion of oxidase activity of myoglobin through the effect of sodium benzoate.

O_2-Uptake was measured during 30 minutes with increasing benzoate concentrations.

The promotion of oxidase activity is expressed in percentage increase of O_2-uptake to the control experiment without benzoate.

The addition of salicylate and dodecylsulfate caused an effect similar to that of benzoate. The concentrations of the perturbators, however, had to be higher to be effective to myoglobin than to hemoglobin. This seems to be due to the stronger resistance of protein moiety of myoglobin than that of hemoglobin against denaturation in general.

II. Determination of Molar Extinction of Verdoheme

The optical constants of protoheme, choleheme and biliverdin have so far been well determined as shown in Table I. As for verdoheme, its spectroscopical constant has not been accurately determined as yet. Based on the experimental results shown in Fig. 1, the molar extinction coefficient could be determined in the following manner.

From the extinctions ε_{573}, ε_{630} and ε_{760} obtained in Fig. 1, the actual increase and decrease of the individual reaction components, protoheme (x), choleheme (y) and verdoheme (z) during the reaction process can be determined by applying the empirical equations presented in our previous paper (*1*). The changes in the extinction of these individual components (x, y and z) during the reaction process could thus be demonstrated as shown in Fig. 4. Among these, the molar concen-

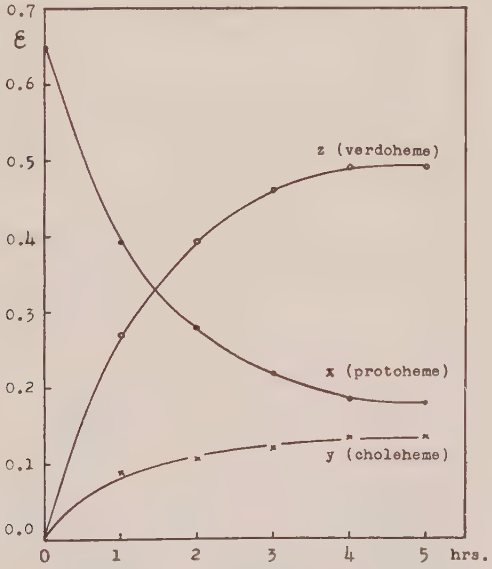

FIG. 4. Changes in the extinctions of protoheme, choleheme, and verdoheme during the course of the reaction.

trations of only protoheme and choleheme can be calculated by applying the constants given in Table I and shown in Fig. 5. If the reaction proceeds without any of the side-reactions, the concentration of verdoheme should be given as follows:

Verdoheme = total heme started − (protoheme remained + choleheme formed)

Now, the concentrations of verdoheme obtained from this equation were plotted against the optical density of the respective z component at 760 mμ (Fig. 6). As shown in Fig. 6, this relation was a straight line indicating that the reaction proceeded without any side reaction as assumed above. By the extrapolation of this straight line in Fig. 6, the molar extinction of verdoheme at 760 mμ was determined as 1.7×10^4. Introducing this value to x component found in the reaction solution (Fig. 4), its absolute concentration was obtained as shown in Fig. 5. Thus it became possible to follow up the whole process of heme breakdown quantitatively in the present reaction system under any of the conditions described.

From the study of the whole process of each component shown in Fig. 5, it became without doubt that the reaction proceeded in the following sequence: Protoheme→choleheme→verdoheme.

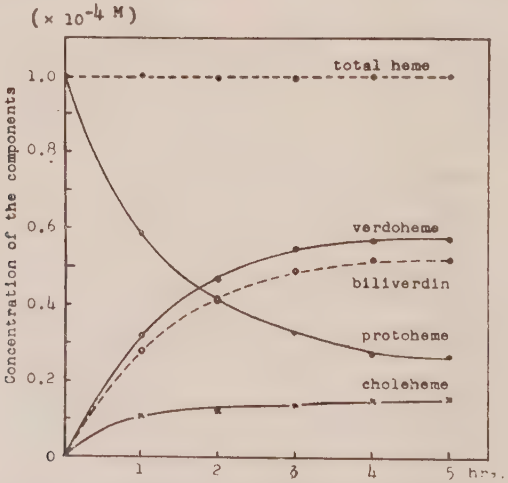

Fig. 5. Changes in each of the components in the reaction solution expressed in terms of absolute concentration. (Calculated from Fig. 4).

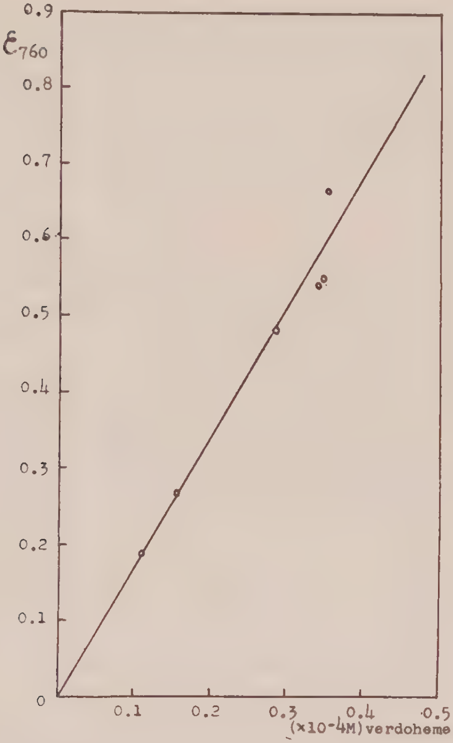

FIG. 6. Calibration curve for verdoheme.

Applying a similar calculation to Fig. 8 in the previous paper, each of the reaction components during the reaction process without the presence of benzoate was obtained as their absolute concentrations (Fig. 7).

III. The Relation between the Amount of Verdoheme Produced and the Yield of Biliverdin

A series of experiments were conducted under conditions similar to that described in Fig. 1 and the verdohemoglobin produced was converted by acid to biliverdin which was then extracted with ether from the reaction solution by the method described in our previous report. The yield of biliverdin was followed up with reaction time. The course of the process is indicated by broken line in Fig. 5. The curve

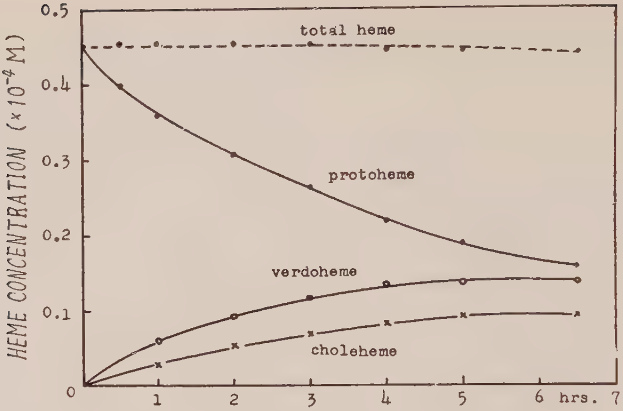

FIG. 7. Changes in each reaction component during the reaction process without benzoate.

(The reaction condition and the procedure of the measurements are the same as those described in Fig. 3 of the previous paper).

of biliverdin obtained agreed fairly well with that of increase in verdoheme, but not with that of choleheme. It became thus evident that the immediate precursor of biliverdin is verdohemoglobin and not choleglobin.

IV. Effect of Ascorbate Concentration upon the Reaction of Verdohemoglobin Formation

Using myoglobin as the starting material and in the presence of benzoate, the reaction was carried out with the addition of ascorbate in varying concentrations. The rate of protoheme breakdown increased with the increasing concentration of ascorbate and in parallel with the increase in the rate of verdoheme formation (Fig. 8). In lower concentrations of ascorbate, the reaction was interrupted more or less at the earlier stage and this is due to the consumption of ascorbate. When ascorbate concentration was estimated after 5.5 hours in the deproteinized reaction solution by the iodine method, the remaining ascorbate was found to be significantly small, as indicated in Table II. In the reaction described in Table II, the initial concentrations of ascorbic acid were taken as 40, 60, 100 and 150 times greater than the concentration of myoglobin. The calculated consumption of ascorbic acid per mole of decomposed protoheme is shown in the same Table. As seen in this table, about 80–100 moles of ascorbic acid were consumed per mole of protoheme broken down. This value is much higher than that in the reaction with hemoglobin, in which the value was calculated as about 20 moles (*4*). This seems to be due to the lower rate of breakdown process of myoglobin compared with that of hemoglobin.

TABLE I

Spectrophotometric Constants of Protoheme, Choleheme, and Biliverdin

	absorption max., mμ	ε_{mM}
Alkali-denatured globin protohemochrome (*4*)	557	30.6
Alkali-denatured globin cholehemochrome (*5*)	618	13.6
CO-compound of protomyoglobin (*3*)	573	12.75
CO-compound of cholemyoglobin	630	16.0
Biliverdin-HCl (*6*)	665	28.6

TABLE II

Initial concentrations of ascorbic acid (*mg./ml.*)	Ascorbic acid / Myoglobin	Ascorbic acid remaining after 5 hrs. (*mg./ml.*)	Protoheme decomposed after 5 hrs. ($\times 10^{-4} M$)	Ascorbic acid consumed / Protoheme decomposed
2.64	150	1.837	0.39	116
1.76	100	0.844		139
		1.056	0.375	106
1.056	60	0.528	0.36	82
0.704	40	0.1425		112
		0.264	0.285	87

V. Experiment with Hemoglobin

In comparison with the reaction starting from myoglobin, a series of experiments were conducted with hemoglobin. Changes of the components in the reaction solution, namely, of protoheme, choleheme, verdoheme and biliverdin (obtained by acid extraction) were recorded in absolute concentrations. Figs. 9 and 10 are the results obtained in the presence and absence of benzoate, respectively.

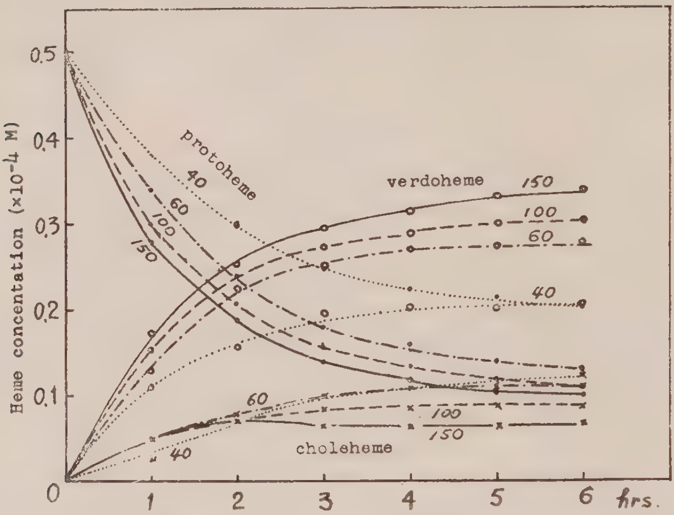

FIG. 8. Changes in each component in the reaction solution with the changes in ascorbate concentration.
Myoglobin 2×10^{-4} *M* 2.5 ml.
Sodium benzoate 0.5 *M* 2.0 ml.
Ascorbic acid (neutralized) 0.5 ml.
T=37°, pH 7.0
The numbers denoted in the figure means the relative multiple of the ascorbate concentration against the concentration of protoheme.

What is common to Figs. 9 and 10, in contrast to the reaction with myoglobin, is a remarkably low production of verdoheme component in spite of a marked increase in choleheme component. In accord with this finding, the yield in biliverdin extractable from this solution was also found to be very low. This is, as described in the previous paper (*5*), due to the intermediate formation of choleglobin-b in the case of the reaction with hemoglobin instead of myoglobin. It is notable, in comparing Fig. 10 with Fig. 9, that choleheme component showed a still higher value and that verdoheme and the yield in biliverdin remained far lower. This indicates that the addition of benzoate

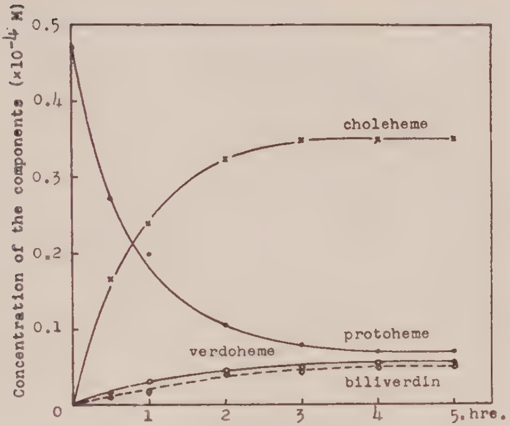

FIG. 9. Changes in each component in the reaction solution starting from hemoglobin.

The absorption was measured after the addition of 12 ml. 1% NaOH and a small amount of powdered $Na_2S_2O_4$, and at each time interval.

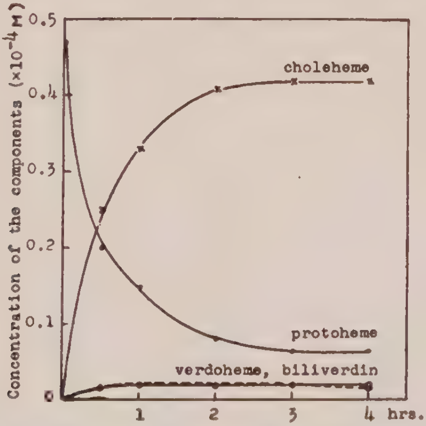

FIG. 10. Hemoglobin breakdown in the presence of benzoate.

Hemoglobin, 3×10^{-4} *M* 2.5 ml.
Ascorbic acid, 0.1 *M* 0.5 ml.
Sodium benzoate, 1.0 *M* 2.0 ml.
T=37°, pH 7.0

is effective in suppressing the conversion of choleglobin into verdohemoglobin. This is due to the promoted formation of choleglobin-b in the presence of benzoate.

It has been reported by Kajita (*6*) in our laboratory that benzoate affects hemoglobin to the unfolding of the molecule (perturbation in the meaning of Holden (*7*)) so that the SH groups in the inner part of the molecule may become exposed. In the reaction system with benzoate added the choleheme produced would be more easily accessible to thus exposed SH groups in the protein moiety to form choleglobin-b. Through the results shown in Fig. 10, the assumption (*8*) that choleglobin-b is the compound of choleglobin combined with protein-SH seems to have gained further support.

DISCUSSION

I. Possibility of Side-reaction

In each of the Figs. 5 to 10, additional data have been given for the total value of heme, the sum of the each component, protoheme, choleheme, and verdoheme. The value of the total heme at every time interval thus given in each of the figures agreed fairly well with the initial value of protoheme. After a longer duration of reaction time (over 7–8 hours), however, the total heme concentration always decreased from the level of initial protoheme. The values of total heme have shown a tendency to decrease after 2 hours, especially in the case of myoglobin reaction and without benzoate (Fig. 7). Some kind of a side-reaction may have occurred at that time.

The most probable side-reaction occurring in this system would be a further decomposition of verdohemoglobin, in whose molecule the porphyrin moiety is converted into biliverdin structure. This possibility may be supported by the following findings. After a longer time intervals, free biliverdin has been detected in the reaction solution as reported in the previous paper. Further, verdohematin prepared from verdohemochrome is stable even in the air, so far as it is kept alkaline. In the neutral solution, however, it undergoes autoxidative decompositions and a pink-colored substance could then be extracted after a treatment with hydrochloric acid. In most cases, the reaction of verdohemoglobin is carried out in a nearly neutral solution so that it seems probable that further oxidation takes place after a longer time intervals. In accord with this assumption, when the reaction was carried out longer, a pink colored substance could be detected by the procedure giving free biliverdin from the reaction mixture.

The second possible side-reaction is that by detachment of iron atom from choleheme, a new porphyrin which might be termed "choleporphyrin" may form in the reaction solution. For instance, when the reaction solution, starting from myoglobin and after a longer reaction time, was treated with glacial acetic acid followed by shaking with ether to extract biliverdin and verdoheme, the remaining water phase was proved to be Gmelin-positive. On the boundary surface of the solution in contact with nitric acid solution, a blue color of biliverdin could distinctly be observable. In this case, the blue color appeared more strikingly on the contact surface, differing from ordinary Gmelin reaction of bilirubin. Possibly the porphyrin, in which its α-methene has been partially oxidized followed by the detachment of iron atom, is further oxidized through Gmelin reaction at the oxidized α-methene group so that the ring structure is cleaved open. A similar color reaction was observed also with choleglobin-b, assumed to have a similar structure as that of porphyrin now in discussion, obtainable from the reaction solution starting from hemoglobin.

II. Reaction Process to Verdohemoglobin from Choleglobin

It is to be noted in Fig. 8 that, when the initial concentration of ascorbic acid was low, so that the reaction was interrupted rather in the earlier stages, the component choleheme formed was found to be relatively higher in value. From this, it may plausibly be assumed that the process from choleglobin to verdohemoglobin also requires the presence of ascorbate. In an earlier report (*9*), dealing with the reaction of verdohemochrome formation from pyridine hemin under similar conditions as in the present reaction, it has been proved that an intermediate product 630-substance underwent decomposition to verdohemochrome (an immediate precursor of biliverdin) consuming molecular oxygen without the coexistence of ascorbate.

As indicated above, however, the reaction from choleglobin to verdohemoglobin seems to proceed under the participation of a reducing substance such as ascorbic acid. Thus, a more precise examination is required for a further elucidation of the reaction mechanism in this stage.

SUMMARY

By the use of the method described in the present paper, it became

possible to follow up quantitatively the reaction process of the formation of verdohemoglobin from hemoglobin or myoglobin through an intermediate choleglobin. The following summarizes the present investigation.

1. The decomposition of myoglobin is promoted by the addition of perturbators as in the case of hemoglobin reaction.

2. The molecular extinction coefficient of verdoheme was determined as $\varepsilon_{\mathrm{mM}}^{760}=1.7\times10^4$, in the state of alkali-denatured globin verdohemochrome.

3. The formation of verdoheme and the yield in biliverdin were found to increase with reaction time in parallel.

4. The reaction of verdohemoglobin formation from myoglobin is promoted by increasing concentrations of ascorbic acid added. In the present reaction, 80–100 moles of ascorbic acid was consumed per mole of myoglobin-heme decomposed.

5. Some side-reactions occur after a longer reaction time.

6. The formation of choleglobin-b during the reaction of verdohemoglobin formation starting from hemoglobin increases when perturbators such as benzoate is present in the reaction system. Accordingly, the formation of verdoheme and consequently the yield in biliverdin were found to decrease in the system mentioned.

The present work was aided by the Grant in Aid for Scientific Research from the Ministry of Education, for which our gratitude is due.

REFERENCES

(*1*) Kaziro, K., Kikuchi, G., and Hanaoka, C., *J. Biochem.*, **42**, 423 (1955)
(*2*) Kikuchi, G., and Tomimura, T., *J. Biochem.*, **41**, 503 (1954)
(*3*) Tsushima, K., and Okazaki, T., *J. Jap. Biochem. Soc.*, **26**, 232 (1954)
(*4*) Kaziro, K., Kikuchi, G., Ogawa, T., and Yamada, M., *J. Biochem.*, **40**, 205 (1953)
(*5*) Hanaoka, C., *J. Jap. Biochem. Soc.*, **26**, 230 (1954)
(*6*) Kajita, A., *J. Biochem.*, **43** 243 (1956)
(*7*) Holden, H. F., *Australian J. Exptl. Biol. Med. Sci.*, **25**, 47 (1947)
(*8*) Lemberg, R., Foulkes, E. C., and Purdom, P., *Proc. Roy. Soc.* (*London*), *B.*, **138**, 386 (1951)
(*9*) Kaziro, K., and Kikuchi, G., *J. Biochem.*, **38**, 213 (1951)

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LIPIDE METABOLISM OF MYCOBACTERIUM TUBERCULOSIS AVIUM

I. ENZYMATIC SYNTHESIS OF HYDROXAMIC ACID FROM FATTY ACID AND ITS SPECIFICITY

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(Received for publication, January 11, 1956)

It is a well known fact that *Mycobacterium tuberculosis* has high content of lipides as cell components. It is expected that the lipide metabolism may play an important role in this bacillus. Many previous reports support this hypothesis (*1–5*).

On the other hand it has been recently clarified that fatty acid oxidation is initiated by the conversion to the corresponding acylated CoA derivative, and the degradation of which is catalyzed by the enzymes of fatty acid cycle proposed by F. Lynen (*6*). From a recent study of acetokinase of *Escherichia coli* (*7*) it became apparent that the enzymatic formation of hydroxamic acid required the participation of ATP.

In our laboratory, fatty acid metabolism of tubercle bacillus has been studied for several years. The present report deals with the activation process of fatty acids: it is shown that the hydroxamic acid forms enzymatically from fatty acid. The results obtained show that *n*-butyric and *n*-valeric acids are more active than acetic acid as substrate of hydroxamic acid formation.

EXPERIMENTAL

Methods and Materials

Assay Systems—

1) The activity of the enzyme was expressed as the amount of hydroxamic acid formed by the method of Lipmann and Tuttle (*8*). A solution containing the

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The following abbreviation will be used throughout this paper: CoA, coenzyme A; ATP, adenosine triphosphate.

ferric complex of 1 μM acetohydroxamic acid per ml. in a cell of 1 cm. light path had an optical density of 1.03 in our experiment. Water-insoluble hydroxamic aicd was determined by the reagent consisting of alcoholic ferric chloride solution (5 per cent $FeCl_3$ in absolute ethyl alcohol).

(2) By coupling with an acetyl acceptor enzyme, acetyl sulfanilamide was determined by the Bratton-Marshall method (*9*).

The values indicated in each case were corrected for the control values in the absence of substrate. The hydroxamic acid formation in most of the controls was from 0.5 to 0.1 μM.

Materials Used—The fatty acids were obtained from commercial sources. For most of the experiments 1.0 or 0.1 *M* solutions were prepared, made neutral by alkali. Crude CoA was prepared by the method of Stadtman and Kornberg (*10*); a unit of the CoA assayed by the Kaplan and Lipmann method (*11*) was 6.5 per mg. The ATP was obtained from Biochimica, A. G., (Zurich).

Preparation of Enzyme Solution—Mycobacterium tuberculosis avium (strain Takeo) was grown in glycerol-bouillon medium for 3 days. The washed cells were frozen and powdered by dry-ice, and then poured into 20 volumes of acetone previously cooled to −30°. After brief stirring, the cells were allowed to settle, the supernatant fluid was desiccated in vacuo until the solvent was completely evaporated.

A portion of these acetone-dried cells was then finely ground and powdered by 4 portions of quartz sand and subsequently extracted with 50 volumes of 0.02 *M* sodium bicarbonate. After aging over night in an ice-box, the cells were centrifuged off at 3000 r.p.m. for 30 minutes, and the supernatant fluid was recentrifuged at 10,000 r.p.m. for 20 minutes at O°. The supernatant fluid was used as crude extract. The enzyme solution was stable indefinitely in the frozen state.

RESULTS

Properties of Enzyme

Coenzyme A Content in Crude Extract—The crude extract prepared from dried cells contained a fairly large amount of CoA. According to the results of assay by the Kaplan and Lipmann's method (*11*), 1 g. of dried cells had about 70 units. Tubercle bacillus had a relatively larger amount of CoA in comparison with other organisms (*cf.* Kaplan *et al.* (*11*)).

Incubation Time Dependence—When the reaction mixture was incubated at 38° with hydroxylamine as trapping agent, hydroxamic acid formation increased as a function of time. The attainment of equilibrium is illustrated in Fig. 1.

Influence of Fatty Acid Concentration on Formation of Hydroxamic Acids—The data summarized in Table I show that the rate of hydroxamic acid

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Page	Line	For	Read
534	1	droxyde,	droxide,
,,	2	slution	solutian
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,,	13	methode	method
,,	22	Fig. 6)	Fig. 5)
535	explanation of Fig. 1.	endogeneous	endogenous
	,,	vloume	volume
537	explanation of Fig. 4.	endogene-	endogen-
,,	TABLE I	Drrk	Dark
538	TABLE III	accumlated	accumulated
539	explanation of Fig. 5.	endgeneous	endogenous
541	3	of	or
,,	4	illuminate.	illuminated.
542	19	place in	placed in
543	3	(*Fraction ii*)	(*Fraction i*)
,,	explanation of Fig. 8.	(corsses)	(crosses)
544	8	lactated,	lactate,
,,	31	racioactive	radioactive
545	TABLE VII	C^{14}-*lacbeled*	C^{14}-*labeled*
,,	,,	Peter. ether,	Petr. ether,
547	10	was	was
549	5	not on the	not in the
550	6	(Figs. 1, 3, 4 and 6)	(Figs. 1, 3, 4 and 5)
,,	20	Fig. 5	Fig. 6
551	21	constituens	constituents
,,	23	difinite	definite
,,	29	assimilation C^{14}-	assimilation of C^{14}-
552	11	*Pseudomanas saccharophylla*	*Pseudomonas saccharophila*
553	15	homogenously	homogeneously

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Page	Line	For	Read
468	Table I	Adsorbing buffers $N/10$ Sodium ecetate (pH 5.0)	Adsorbing buffers $N/10$ Sodium acetate (pH 5.0)
		Eluting buffers $N/10$ Ammonium acetate (pH 6.0) $N/10$ Sodium acetate (pH 7.1) $N/10$ Ammonium acetate (pH 6.0)	Eluting buffers $5N/10$ Ammonium ace. tate (pH 6.0) $5N/10$ Sodium acetate (pH 7.1) $4N/10$ Ammonium acetate (pH 6.0)
806	18	isobutanol	isopropanol
810	Table I, No. 7,8	[Var]	[Val]

548 Table IX is to be read as follows:

TABLE IX

	Gas phase filled with	Time	C^{14} incorporated in	
			TCA-precipitated cells	CO_2
Dark	5% CO_2 in air	30 *min.*	43 *c.p.m.*	238 *c.p.m.*
		60	108	598
	CO_2 free air	30	93	249
		60	118	552
Light	5% CO_2 in air	30	185	198
		60	277	319
	CO_2 free air	30	199	185
		60	296	285

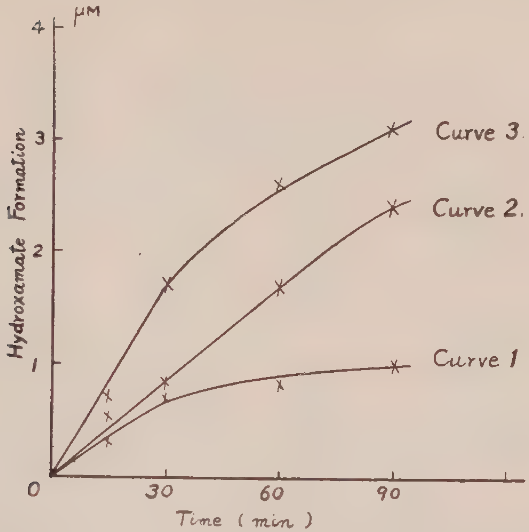

FIG. 1. Rate of hydroxamic acid formation as a function of time. The reaction mixture contains: crude extract, 2.0 ml.; $NaHCO_3$, 40 μM; NH_2OH, 1000 μM; acetate (Curve 1), succinate, (Curve 2), 100 μM; sebacinate (Curve 3), 10 μM. Total volume, 3.7 ml. 38°, pH 6.8. Incubation time as indicated.

formation increases with substrate concentration at any given enzyme concentration. The affinity of each fatty acid for the enzyme will be determined as Michaelis constant by the purified enzyme in the later reports. It is at present evident that any hydroxamic acid is not formed from aspartic acid, glutamic acid, valine, leucine, asparagine, or glutamine.

Heat Inactivation—When the crude extract was heated for 15 minutes at 60° and 80°, the enzyme lost approximately 86 and 100 per cent of its activity respectively. This enzyme is much more thermolabile than heat-resistant esterase obtained from the same strain by Yamamura *et al.* (*12*).

Capacity of Various Fatty Acids to Form Hydroxamic Acids

Influence of Chain Length of Saturated Fatty Acid—Fig. 2 shows the results of a gross comparison of a series of various saturated fatty acids

TABLE I

Substrate Concentration

The reaction mixture contains: crude extract, 2.0 ml.; $NaHCO_3$, 40 μM; NH_2OH, 1000 μM; substrate as indicated. Total volume, 4.0 ml., 38°, 90 minutes, pH 6.8

Substrate	Added	Hydroxamic acid formed
	μM	μM
n-Butyrate	20 50 100	1.66 2.61 3.74
n-Valerate	8 20	1.38 2.95
Succinate	30 100 200	0.89 1.37 1.73
Laurate*	0.5 1 2 4	0.12 0.20 0.31 0.34
Glutamate	50 100	0.06 0.05
Glutamine	10 50 100	0.08 0.14 0.25

* Crude extract, 0.5 ml.; NH_2OH 600 μM; laurate as indicated, 120 minutes. Total volume 1.0 ml.

from formic to pelargonic (C-1∼C-9). Of the acids serving as substrates for this enzyme, *n*-valeric and *n*-butyric acids were the most effective. Branched isovaleric acid also served as a good substrate. However, a little amount of hydroxamic acid was formed with acetic acid, contrary to previous expectation. In a comparison of relative activity, valeric acid was 16 times as active as acetic acid.

Capacity of Dicarboxylic Acids to form the Corresponding Hydroxamic Acids—The results of a gross comparison of a series of dicarboxylic acids from malonic to sebacic (C-3∼C-10) with respect to the rate of hydroxyamic acid formation are shown in Fig. 3. The acids from C-5 to C-7 were inert. The maximal values of the enzyme activity was observed with succinic and sebacic acids of a number of acids tested.

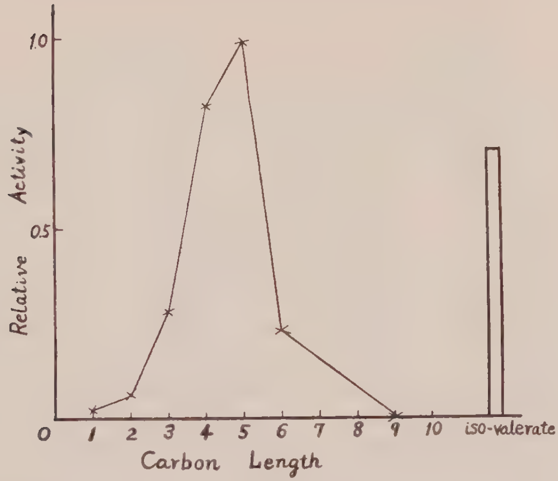

FIG. 2. Influence of chain length of saturated fatty acids.

The reaction mixture contains: crude extract, 2.0 ml.; $NaHCO_3$, 40 μM; NH_2OH, 1000 μM; each substrate, 10 μM. Total volume, 4.0 ml. 38°, 60 minutes, pH 6.8. (Relative activity is defined as 1.00 when 10 μM *n*-valerate forms 0.98 μM hydroxamate under the above conditions.)

Capacity of Higher Fatty Acids to Form Hydroxamic Acids—The data of a similar comparison as in Figs 2 and 3 are presented in Fig. 4. Of the higher fatty acids from lauric to stearic (C-12$\sim$C-18), and oleic and linoleic acids (C-18), lauric, myristic, oleic and lionoleic acids were the most effective. Noteworthy is the fact that unsaturated C-18 acids were 3 times as active as saturated C-18 acid.

Enzymatic Acetyl Transfer to Sulfanilamide

The crude extract catalyzed the reaction of acetic acid with sulfanilamide to form acetyl sulfanilamide. Free sulfanilamide was determined with aliquots of the incubation mixture by the Bratton-Marshall diazotization procedure. Acylation of sulfanilamide took place when formic and acetic acids were used as the substrate; however, the rate was relatively slow (Table II). The results summarized from Fig. 2 and Table II indicate that acetic acid does not serve as an effective acetyl donor.

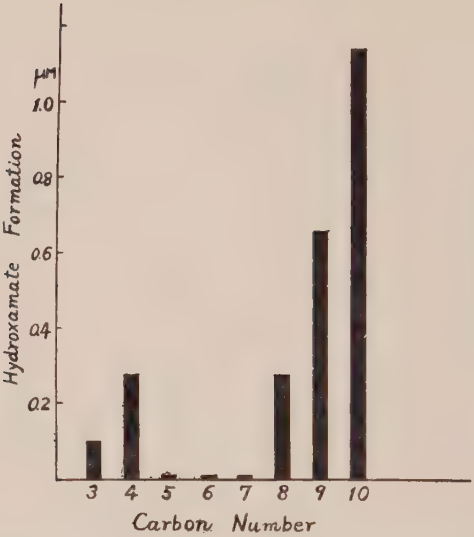

FIG. 3. Capacity of dicarboxylic acids to form the corresponding hydroxamic acid.

The reaction mixture contains: crude extract, 2.0 ml.; $NaHCO_3$, 40 μM; NH_2OH, 1000 μM; each substrate, 10 μM. Total volume, 3.7 ml., 38°, 60 minutes, pH 6.8.

Factors Influencing Hydroxamic Acid Formation and Acetylation of Sulfanilamide

By the analogy of other activation reactions, hydroxamic acid formation should require the participation of ATP, or both ATP and CoA. However, in the case of this bacillus, no addition of ATP and CoA was required. To remove these nucleotides, the charcoal treatment was applied. The activity lost by this treatment was approximately 20～30 per cent of the original activity; however, by the addition of ATP, or of CoA, or both ATP and CoA, the activity was not recovered completely. The need for ATP was not established, although a slight stimulation of the rate was observed by the addition of ATP (Table III).

As shown in Table IV, the participation of ATP and CoA in the acetylation of sulfanilamide was studied. As can be seen from the results, slight increases in acetylation were observed with additions of

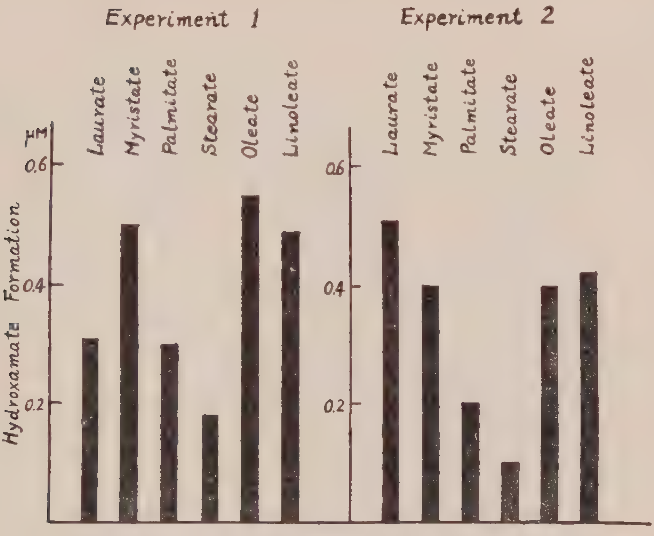

FIG. 4. Hydroxamic acid formation from higher fatty acids.

The reaction mixture contains: crude extract, 0.4 ml.; NH_2OH, 600 μM; each substrate, 6 μM in experiment 1.

The reaction mixture contains: partially purified enzyme (ammonium sulfate precipitation 20–40 per cent in saturation), 0.4 ml.; NH_2OH, 600 μM; each substrate, 6 μM in *Experiment* 2. Total volume, 1.0 ml., 38°, 120 minutes.

Hydroxamic acids were determined by alcoholic $FeCl_3$ reagent (see the text).

ATP or of CoA, or of both. The above results that the added ATP and CoA had only a slight effect on hydroxamic acid formation and on acetylation of sulfanilamide was due to the fact that the crude extract readily contained a fairly large amount of labile phosphate and CoA.

DISCUSSION

Several considerations must be now required in relation to the experimental conditions used. In the first place, the enzymatic formation of ester by the crude extract may be occured ; however, hydroxamic acid is not formed from esters at neutral pH, but highly alkaline conditions being required for the hydroxamic acid synthesis. More-

TABLE II

Enzymatic Acetylation of Sulfanilamide

Experiment No.	Substrate added	Free sulfanilamide		Acylated sulfanilamide (2)−(1)
		Direct (1)	After hydrolysis (2)	
		μM	μM	μM
I	None	1.38	1.43	0.05
	Acetate	1.17	1.40	0.23
	Formate	1.27	1.43	0.16
II	None	1.53	1.54	0.01
	Acetate	1.34	1.54	0.20

(1): Residual free sulfanilamide was determined directly.

(2): 0.2 ml. of 4 *N* HCl was added to 2.0 ml. aliquot of incubation mixture. After hydrolysis at 100° for 60 minutes, total free sulfanilamide was determined by usual method.

(2)−(1): Enzymatically acylated sulfanilamide was calculated by (1) minus (2).

The reaction mixture contains: crude extract 0.5 ml. (Expt. I); 0.8 ml. (Expt. II); each substrate, 100 μM. Total volume 1.5 ml., 38°, 120 minutes, pH 7.6, anaerobic conditions.

TABLE III

Participation of ATP

The reaction mixture contains: partially purified enzyme**, 2.0 ml.; $NaHCO_3$, 40 μM; NH_2OH, 1000 μM; succinate, 100 μM. Complete system contains Na ATP, 5 μM; and CoA, 3.3 units. 38°, 90 minutes, pH 6.8. Total volume, 4.3 ml., anaerobic condition

Addition	Hydroxamic acid formed
	μM
Complete system	3.83
No CoA*	3.44
No ATP*	3.31
No substrate*	0.12

* This means absence from the complete system.

** " Partially purified enzyme " is the supernatant of the charcoal treated and centrifuged portion of the ammonium sulfate precipitated fraction (20–50 per cent saturation) of the crude extract.

over, Lipmann and Tuttle (*13*) reported on the direct hydroxamate

TABLE IV

Participation of ATP and CoA for Acetylation of Sulfanilamide

The reaction mixture contains: crude extract, 0.78 ml; $NaHCO_3$, 40 μM; if added, CoA, 6.5 units; Na ATP, 5 μM; acetate, 100 μM Total volume, 1.5 ml. 38°, 60 minutes, pH 7.4.

Addditions	Without incubation			With incubation		
	Free sulfanilamide		Acetylated sulfanilamide (2)−(1)	Free sulfanilamide		Acetylated sulfanil-amide (2)−(1)
	Direct (1)	After hydrolysis (2)		Direct (1)	After hydrolysis (2)	
	μM	μM	μM	μM	μM	μL
None	1.36	1.37	0.01	1.29	1.37	0.08
Acetate	1.39	1.35	0.00	1.18	1.34	0.16
CoA	1.36	1.37	0.01	1.25	1.37	0.12
Acetate+CoA	1.29	1.33	0.04	1.13	1.35	0.22
ATP	1.32	1.37	0.05	1.36	1.32	0.00
Acetate+ATP	1.29	1.35	0.06	1.20	1.34	0.14
CoA+ATP	1.39	1.33	0.00	1.27	1.37	0.10
Acetate+Co#+ATP	1.27	1.33	0.06	1.15	1.37	0.22
None (with heated enzyme)*	1.29	1.32	0.03	1.23	1.32	0.09
Acetate (”)*	1.29	1.32	0.03	1.23	1.32	0.09

* enzyme was heated for 15 minutes at 80°.

formation from fatty acid with lipase. However, the fact that esterase of avian tubercle bacillus is more thermostable than the enzyme in present study, will strictly distinguished between both enzyme activities.

In the second place, carboxylic acid or acid amide will be converted to the corresponding hydroxamic acid by amidase or transpeptidase. When those activities were measured by the hydroxamic acid method, there was no detectable formation of hydroxamic acid from aspartic acid, glutamic acid, valine, leucine, glutamine, or asparagine. Therefore, it can certainly be concluded that the hydroxamic acid formation is negligibly low under these conditions with amidase or transpeptidase.

In the third place, the possiblity of acylhydroxylamine kinase can be excluded for the reason that high hydroxylamine concentration is not inhibitory in the crude extract. Since the partially purified enzyme contains a fairly large amount of undialyzable labile phosphate which is hydrolyzed only chemically or enzymatically, it is concluded that the measured hydroxamic acid formation corresponds to the activation reaction of fatty acids. Whether this activation is due to the presence of kinase or CoA-kinase can not be concluded from the present hydroxylamine trapped experiments.

The activation pattern of fatty acids by the tubercle bacillus was observed to be specific in comparison with previously reported data obtained in the studies on animal tissues (*14*), (*15*), yeast (*16*), and *Escherichia coli* (*17*). It is apparent that this specificity will have an important significance in lipide metabolism, especially higher fatty acid synthesis of tubercle bacillus. The interpretation of these comparison is limited by the fact that experimental conditions were not optimal to obtain rates for each fatty acid.

SUMMARY

1. The conversion of fatty acids to the corresponding hydroxamic acid was demonstrated with crude extract from Mycobacterium tuberculosis avium (strain Takeo).

2. Of the acids serving as substrates for this enzyme, straight saturated butyric and valeric, dicarboxylic sebacic, and unsaturated oleic acids were the most effective. Marginal formation of hydroxamic acid was observed with formic, glycolic, glyoxylic, pelargonic, caprinic, capronic, glutaric, adipic, and pimelic acids, and with several amino acids.

3. The hydroxamic acid formation is strongly supported by the present data as the carboxyl activation. It is also evident that this formation is not esterase-, amidase-, or transpeptidase-catalyzed reaction.

4. All the results summarized in the present study can be explained by the following reactions:

$$\text{Fatty acid}+\text{labile phosphate (protein bound)}\longrightarrow$$

$$\text{(acyl-phosphate intermediate)}\xrightarrow{NH_2OH}\text{hydroxamic acid.}$$

The detailed function of protein bound labile phosphate remains to be clarified.

The authors are indebted to Mr. H. Katayama and Mr. J. Tobari for technical assistance, and to Prof. Dr. S. Akabori for his valuable advice and encouragement during the cource of this study.

REFERENCES

(*1*) Cutinelli, C., *Boll. ist. sieroterap. milan.*, **19**, 88, 141, 146 (1940)

(*2*) Franke, W., and Schillinger, A., *Biochem. Z.*, **316**, 313 (1944)

(*3*) Yamamura, Y., Sasakawa, T., and Imazu, S., *Kekkaku*, **24**, 435 (1949)

(*4*) Oginsky, E. L., Smith, P. H., and Solotorovsky, M., *J. Bacteriol.*, **59**, 29 (1950)

(*5*) Lindsay, M., O'Donnel, T. V., and Edson, N. L., *Biochem. J.* **46**, 248 (1950)

(*6*) Lynen, F., *Ann. Rev. Biochem.*, **24**, 653 (1955)

(*7*) Rose, I. M., Grunberg-Mango, M., Korey, S. R., and Ochoa, S., *J. Biol. Chem.*, **211**, 737 (1954)

(*8*) Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159**, 21 (1945)

(*9*) Bratton, A. C., and Marshall, E. K., *J. Biol. Chem.*, **128**, 537 (1939)

(*10*) Stadtman, E. R., and Kornberg, A., *J. Biol. Chem.*, **203**, 47 (1953)

(*11*) Kaplan, N. O., and Lipmann, F., *J. Biol. Chem.*, **174**, 37 (1948)

(*12*) Yamamura, Y., Ogura, K., and Imazu, S., *Kekkaku*, **28**, 51 (1953)

(*13*) Lipmann, F., and Tuttle, I. C., *Biochim. et Biophys. Acta.*, **4**, 211 (1950)

(*14*) Kornberg, A., and Pricer, W. E., *J. Biol. Chem.*, **204**, 329 (1953)

(*15*) Hele, P., *J. Biol. Chem.*, **206**, 671 (1954)

(*16*) Jones, M. E., and Lipmann. F., Methods in enzymology, Academic Press Inc., New York (1955), Vol. I, p. 585.,

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THE CHANGE OF *N*-SUBSTITUTED AMINO ACIDS IN THE ANIMAL BODY

XIV. THE FATE OF C^{14}-METHYL GROUP ATTACHED TO NITROGEN OF AMINO ACID

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(Received for publication, January 13, 1956)

The fate of *N*-substituted amino acid, especially its C-skeleton, in the animal body has been repeatedly studied (*1–6*, *10–13*) in our laboratory. The oxidation of methyl group which attached to N of an amino acid has also been studied (*7–9*, *14*, *15*) and it has disclosed that it is oxidized into HCHO by the action of demethylase existing in a very active form in rabbit kidney. The present communication deals with the further fate of it in the rabbit body as investigated by using *N*-$C^{14}H_3$-L-phenylalanine.

As the preliminary step for the synthesis of experimental material the correspondingly labeled methyl iodide was synthesized, since *N*-methylphenylalanine can be conveniently prepared by the condensation of methyl iodide with *p*-toluenesulfonyl-L-phenylalanine by Fischer's method (*16*). The synthesis of methyl iodide was accomplished through the following series of reactions using radioactive barium carbonate.

$$BaCO_3 \xrightarrow{HClO_4} CO_2 \xrightarrow{LiAlH_4} CH_3OH \xrightarrow{\text{red } P+I_2} CH_3I$$

In the present experiment 1 g. of radioactive *N*-methyl-L-phenylalanine was administered by intraperitoneal injection to a rabbit (800 g.) previously kept fasting for 24 hours, and the radioactivity of the CO_2 expired within 24 hours and some methyl compounds contained in the carcase were measured.

EXPERIMENTAL

Assay of Samples for C^{14}—Samples were first converted by the "wet" method of Peters (*17*) into carbon dioxide which was absorbed in sodium hydroxide solution, and then precipitated by addition of a sufficient amount of 30 per cent $BaCl_2$ (usually

2 ml.). The precipitate was collected by filtration on a filter paper disk 3.14 sq. cm. in area. The $BaCO_3$ disk was left drying for 5 minutes under an infra-red lamp. The radioactivity of the $BaCO_3$ samples was measured by a Geiger-Muller counter (Nuclear) with a mica window.

Synthesis of N-Methyl-L-phenylalanine—i) *Methanol* $C^{14}H_3OH$: One mC of $BaCO_3$, diluted with 6.5 g. of ordinary barium carbonate, was poured quantitatively into an 100 ml.- flask, to which was added 10 ml. of water. 25 ml. of 60 per cent perchloric acid was charged into a separatory funnel inserted into the rubber stopper of the flask. The flask was chilled till the content was frozen in a methanol bath containing dry ice. The perchloric acid was slowly dropped into the flask and the reaction began as soon as the ice in the flask melted. The generated carbon dioxide was diluted with a stream of nitrogen, and passed through 250 ml. of $LiAlH_4$ (1.9 g.) solution in diethyleneglycol diethyl ether. When the passage of nitrogen gas through the solution had been continued for 1 hour, 60 g. of diethyleneglycol monobutyl ether was added and the bubbling with N_2-gas was further continued for another one hour, the mixture being heated in an oil-bath at 110°. The product was collected at 70 mm. -Hg pressure, in a trap cooled in a liquid-air bath. The yield was 1.05 g. or about 100 per cent.

ii) *Methyl Iodide*, $C^{14}H_3I$: The methanol prepared as stated above was diluted to 2 ml. with ordinary methanol and converted to methyl iodide by the method of Tolbert (*18*). The boiling point of redistilled methyl iodide was 42 to 43°, and the yield 3.65 g.

iii) *p-Toluenesulfonyl-L-phenylalanine*, $C_6H_5CH_2CH(NC^{14}H_3SO_2C_7H_7)COOH$: 4.14 g. of *p*-toluenesulfonyl-L-phenylalanine, prepared by Fischer's method (*16*) and melted at 164°, was heated at 70° with 78 ml. of *N*/2 sodium hydroxide and 3.64 g. of methyl iodide in a sealed tube. The resulting product was recrystallized from benzol and petroleum ether, the yield reaching 4.2 g. and melting at 92 to 93°.

iv) *N-Methyl-L-phenylalanine*, $C_6H_5CH_2CH(NHC^{14}H_3)COOH$: 4.14 g. of *p*-toluenesulfonyl-L-methylphenylalanine was dissolved in about 18 ml. of glacious acetic acid and hydrolyzed with 9 ml. of concentrated hydrochloric acid in a sealed tube at 100° for 16 hours. *N*-Methyl-L-phenylalanine, recrystallized from boiling water, had m.p. 278° (dec.) and $[\alpha]_D^{25}=+49.05$ (in *N*/10 NaOH).

The specific radioactivity of *N*-methyl-L-phenylalanine was 7.28×10^3 c.p.m./mg. The over-all yields of radio carbon, after conversion of barium carbonate to *N*-methyl-L-phenylalanine, was 36.4 per cent.

The oxygen uptake of this methylamino acid in the presence of rabbit kidney demethylase is shown in Fig. 1.

Animal Experiment—One g. of the labeled *N*-methyl-L-phenylalanine was administered by intraperitoneal injection to a rabbit (800 g.) kept fasting for 24 hours. Immediately after the administration the animal was placed in a metabolism apparatus designed for the continuous and quantitative collection of expired CO_2, and its radioactivity, as measured according to Mackenzie *et al.* (*19*), is shown in Fig. 2. Food was withheld during the remainder of the experiment.

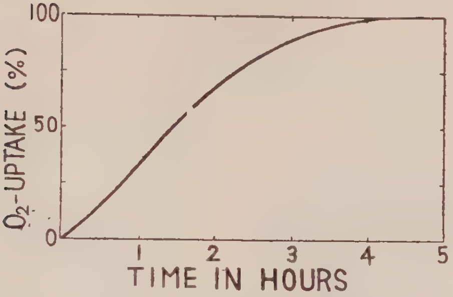


FIG. 1. 1 ml. of 0.01 *M* solution of *N*-$C^{14}H_3$-L-phenylalanine, 0.5 ml. of centrifuged aqueous extract of the aceton powder of rabbit kidney (1:10) in 1.0 ml. of *M*/15 phosphate buffer (pH 7.2).

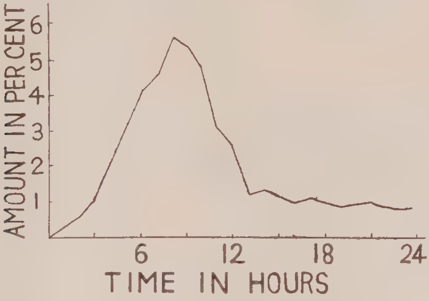


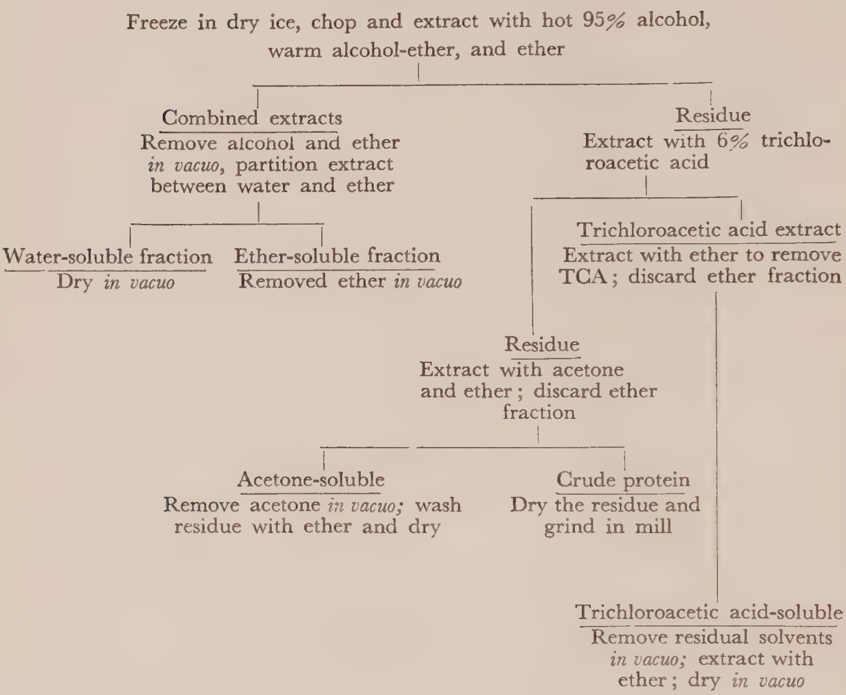
FIG. 2. The curve of the amount of methyl carbon accounted for in the carbon dioxide expired per hour.

24 hours after the injection of amino acid the rabbit was anesthetized to death with ether, skinned, and dissected. The intestinal tract was washed out with water and the muscles were removed from the bones. The muscles and viscerae were chopped and extracted by the procedure illustrated in Diagram 1. Several extractions were carried out with each solvent. Choline was isolated in the form of choline chloroplatinate from both the water and ether soluble fractions; creatine contained in both the water and trichloroacetic acid soluble fractions was converted into creatinine and isolated as its potassium picrate double salt by the method of du Vigneaud *et al.* (*20*); methionine was isolated from both the trichloroacetic acid soluble fraction and the crude protein fraction by Hill's method (*21*); histidine from the crude protein fraction according to Vickery (*22*); purine as cuprous salt from both the trichloroacetic acid soluble fraction and the crude protein fraction by Graff's method (*23*); and creatinine in urine as picrate by the usual method.

The results of the experiments are shown in Tables I and II. Especially, Table

DIAGRAM I

Procedure for Extraction Muscles and Viscera


Freeze in dry ice, chop and extract with hot 95% alcohol, warm alcohol-ether, and ether
Combined extracts
Remove alcohol and ether in vacuo, partition extract between water and ether
Water-soluble fraction
Dry in vacuo
Ether-soluble fraction
Removed ether in vacuo
Residue
Extract with 6% trichloroacetic acid
Trichloroacetic acid extract
Extract with ether to remove TCA; discard ether fraction
Residue
Extract with acetone and ether; discard ether fraction
Acetone-soluble
Remove acetone in vacuo; wash residue with ether and dry
Crude protein
Dry the residue and grind in mill
Trichloroacetic acid-soluble
Remove residual solvents in vacuo; extract with ether; dry in vacuo

II shows in percentage the distribution in counts of radioactivity of each compound as based on administered *N*-methyl-L-phenylalanine.

DISCUSSION

In the present experiment *N*-methyl-L-phenylalanine has been preferred, since rabbit kidney demethylase oxidizes more easily the methyl derivative of aromatic or heterocyclic amino acid than it does that of aliphatic amino acid.

The synthetic methods hitherto used for obtaining labeled methyl iodide from $BaC^{14}O_3$, needed a high vacuum, but in the present experiment pure methyl iodide was obtained with a good yield without it.

The presence of radioactivity in the carbon dioxide expired during the first one hour of the experiment demonstrated that the rabbit was

TABLE I

The Quantity of Compounds Isolated

Compound	Fraction	Amount mg.	Reference of the method of assay
Choline	Ether soluble	745.5 (platinate)	*(21)*
”	Water soluble	264.6 (”)	*(21)*
Creatine	Water soluble	769.2 (K. picrate)	*(24)*
”	T.C.A. soluble	248.5 (”)	*(24)*
Creatinine	Urine	652.2 (Picrate)	*(24)*
Methionine	T.C.A. soluble	79.6	*(25)*
”	Crude protein	618.5	*(25)*
Purine	T.C.A. soluble	248.5 (cuprous salt)	*(23)*
”	Crude protein	315.3 (”)	*(23)*
Histidine	Crude protein	1028.8	*(26)*

TABLE II

Distribution of Radioactivity in Each Compound in per Cent of Counts Based on N-Methylphenylalanine Administrered

Compound	Fraction	%
Carbon dioxide	Expired air	47.27
Choline	Ether soluble	4.178
”	Water soluble	1.612
Creatine	Water soluble	1.033
”	T.C.A. soluble	0.476
Creatinine	Urine	0.205
Methionine	T.C.A. soluble	0.021
”	Crude protein	0.163
Purine	T.C.A. soluble	0.031
”	Crude protein	0.055
Histidine	Crude protein	0.083
Total		55.127

able to oxidize readily the methyl group administered in the form of *N*-methyl-L-phenylalanine, the radioactivity reaching 0.23 per cent of that of injected amino acid. The radioactivity of CO_2 expired per hour rose to a peak value of 5.5 per cent 8 hours after the injection. Thereafter the rate of expiration declined to an average value of 1.0 per cent in 13 to 24 hours.

Within 24 hours 47.27 per cent of the methyl group of the compound administered was completely oxidized to carbon dioxide and expired. This value in per cnt was determined without taking into account the carbon dioxide that was formed from the methyl group subsequently to take part in some synthetic reactions in the body.

An additional 0.205 per cent of the administered methyl group was eliminated in the urine as creatinine. A total of 47.475 per cent of the administered methyl carbon left the body in these two different forms during the course of the experiment. 14.25 per cent of the administered methyl carbon was found remaining in the body as the methyl groups of creatine, choline and methionine, 24 hours after the injection, participating in transmethylation reactions. It was thus confirmed that a part of the formaldehyde, liberated from *N*-methyl-L-amino acid by demethylase, entered into transmethylation reactions in the rabbit body, a finding, which agrees with the observation of Berg showing that formaldehyde becomes the methyl group of methionine (*27*).

According to Greenberg (*28*), formaldehyde is utilized in the synthesis of purine by liver. This fact was also confirmed by the present experiment.

Levy and Coon (*29*) has found that radioactive formate is an excellent precursor of radioactive histidine and that it furnisches only 1 carbon of the molecule, namely the amidine carbon of the imidazole ring. Incorporation of formaldehyde into histidine was observed in the present experiment.

SUMMARY

N-Methyl-L-phenylalanine containing C^{14} in the methyl group was synthesized in order to trace the fate of formaldehyde liberated from it by demethylase in the rabbit body.

One g. of the synthetized compound was given to a rabbit (800 g.). Within 24 hours 47.27 per cent of the methyl group administered was oxidized to carbon dioxide and expired.

There remained still 14.25 per cent of the substance in the body 24 hours afterwards, participating in transmethylation reactions and forming creatine, choline and methionine.

A part of formaldehyde was likely to take part in the formation of purine and histidine in the rabbit body.

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REFERENCES

(*1*) Chin, K., *Z. Physiol. Chem.*, **257**, 12 (1938)

(*2*) Chin, K., *Z. Physiol. Chem.*, **257**, 18 (1938)

(*3*) To, T., *Z. Physiol. Chem.*, **260**, 175 (1939)

(*4*) Rin, K., *J. Med. Assn. (Formosa)*, **319**, 1947 (1940)

(*5*) Fukuyama, S., *J. Biochem.*, **33**, 73 (1941)

(*6*) Yosida, T., *J. Biochem.*, **33**, 319 (1941)

(*7*) Yosida, T., and Fukuyama, S., *J. Biochem.*, **34**, 429 (1941)

(*8*) Yosida, T., and Fukuyama, S., *J. Biochem.*, **36**, 349 (1944)

(*9*) Yosida, T., *J. Biochem.*, **36**, 355 (1944)

(*10*) Fukuyama, S., *J. Japan. Biochem. Soc.*, **18**, 115 (1944)

(*11*) Fukuyama, S., *J. Japan. Biochem. Soc.*, **18**, 336 (1944)

(*12*) Komaki, T., *J. Biochem.*, **37**, 461 (1950)

(*13*) Utsunomiya, J., *J. Biochem.*, **37**, 145 (1950)

(*14*) Moritani, M., Tung, T., Fujii, S., Mito, H., Izumiya, N., Kenmochi, K., and Hirohata, R., *J. Biol. Chem.*, **209**, 485 (1954)

(*15*) Hirohata, R., *J. Japan. Biochem. Soc.*, **26**, 77 (1954)

(*16*) Fischer, E., and Lipschitz, W., *Ber. Chem. Ges.* **48**, 374 (1915)

(*17*) Peters, J. H., and Gutman, H. R., *Anal. Chem.*, **26**, 987 (1953)

(*18*) Tolbert, B. M., *J. Am. Chem. Soc.*, **69**, 1529 (1946)

(*19*) Mackenzie, C. G., Chandler, J. P., Keller, E. B., Rachele, Jr., Cross, N., and du Vigneaud, V., *J. Biol. Chem.*, **180**, 99 (1949)

(*20*) du Vigneaud, V., Cohn, M. C., Chandler, J. P., Schenck, J. R., and Simmonds, S., *J. Biol. Chem.*, **140**, 625 (1941)

(*21*) Hill, F. M., and Robson, W., *Biochem. J.*, **28**, 1008 (1934)

(*22*) Vickery, H. B., *J. Biol. Chem.*, **143**, 77 (1942)

(*23*) Graff, S., and Maculla, A., *J. Biol. Chem.*, **110**, 71 (1935)

(*24*) Folin, O., *J. Biol. Chem.*, **17**, 469 (1914)

(*25*) Horn, M. J., Jones, D. B., and Blum, A. E., *J. Biol. Chem.*, **151**, 635 (1943)

(*26*) Akabori, S., Chemistry of protein, Kyoritsu Shuppan Co., Ltd., Tokyo, Vol. **I**, 238 (1954)

(*27*) Berg, P., *J. Biol. Chem.*, **190**, 31 (1951); *Fed. Proc.*, **11**, 386 (1952)

(*28*) Greenberg, D., *Fed. Proc.*, **10**, 192 (1951)

(*29*) Levy, L., and Coon, M., *J. Biol. Chem.*, **192**, 507 (1951)

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STUDIES ON CHROMOPROTEINS IN JAPANESE NORI (PORPHYRA TENERA)

II. AMINO ACID COMPOSITIONS OF PHYCOERYTHRIN AND PHYCOCYANIN

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(Received for publication, January 16, 1956)

Phycoerythrin and phycocyanin are water-soluble chromoproteins contained in marine algae, and they function in photosynthesis, the mechanisms of which have been investigated in recent years (*1–4*). The author has previously found a convenient method for the separation and purification of these two proteins from Japanese Nori (*Porphyra tenera*) using rivanol (2-ethoxy-6,9-diaminoacridinum lactate), and obtained electrophoretically pure crystalline materials (*5*). Absorption maxima are at 496, 546, 560 mμ for phycoerythrythrin and at 615 mμ for phycocyanin ; the data of elemental analysis are as follows (*4*) :

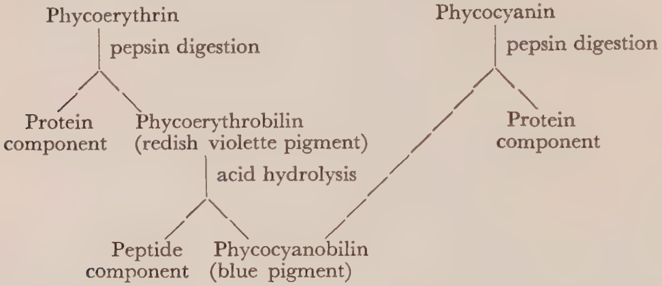
	C	H	N	S	Ash
phycoerythrin (%)	50.80	7.99	15.13	2.06	1.06
phycocyanin (%)	51.01	6.82	15.81	1.12	1.52

These chromoproteins were heretofore studied chiefly from the chromochrmical standpoint ; their prosthetic groups are known to be bile pigments, which combine with the proteins more firmly than in the case of hemoproteins (*6*, *7*, *8*). Kitasato has reported the scheme shown in Fig. 1 and indicated that these two chromoproteins have a similar prosthetic group to phycocyanobilin, which was identified by Lemberg (*7*) as mesobiliviolin. Thus these proteins were found to be closely related to their prosthetic groups. Moreover, close relations are also considered to exist between their protein components, because of the identity of their end groups (*9*).

The author has found the similarity of these protein components, by estimating their amino acid compositions by the ion exchange chromatography according to the technique of Moore and Stein

(*10*, *11*), the results of which are discribed in this paper.

Fig. 1. Scheme for the prosthetic groups of phycocyanin and phycoerythrin (*8*).



METHODS AND MATERIALS

Materials—Phycoerythrin and phycocyanin were prepared by the rivanol method as discribed in the previous author's paper (*4*) from the air dried Japanese Nori (*Porphyra tenera*). They were recrystallized five times from ammonium sulfate solution. Final crystals were dialyzed against distilled water and then lyophilized.

Hydrolysis—Crystalline phycoerythrin and phycocyanin (each 200 mg. to 250 mg.) were hydrolyzed with 15 times the weight of 6 *N* HCl in sealed glass tubes for 24 hours at 105°. After the removal of humin by filtration and excess hydrochloric acid by evaporation under reduced pressure at 40°, the residual products were taken up in a definite volume of proper solvent used for analysis.

Performic Acid Oxidation—Cystine and cysteine were estimated as cysteic acid by the method of Schram, Moore and Bigwood (*12*). The performic acid reagent was prepared by the addition of 1 vol. of 30 per cent (*w.*/*w.*) H_2O_2 to 9 vol. of 96 per cent (*w.*/*w.*) formic acid. In the oxidation of cystine and cysteine, 50 mg. of protein was dissolved in 4 ml. of formic acid, to which was added 8 ml. of performic acid solution, and then the mixture was kept 5° for 4 hours to allow the reaction to proceed.

Systematic Chromatographic Analysis of Amino Acids—Chromatographic determination of amino acids were carried out by Moore and Stein's technique (*10*, *11*) using Dowex-50 or Dowex-2 ion exchange resin columns selectively according to the group of substances as follows:

A. Mono-amino acid group: column 100×0.9 cm. Dowex-50.
 solvent; pH 3.42 citrate buffer at 37.5°
  pH 4.25 citrate buffer at 50.0°
B. Basic amino acid group: column 15×0.9 cm. Dowex-50
 solvent; pH 5.0 citrate buffer

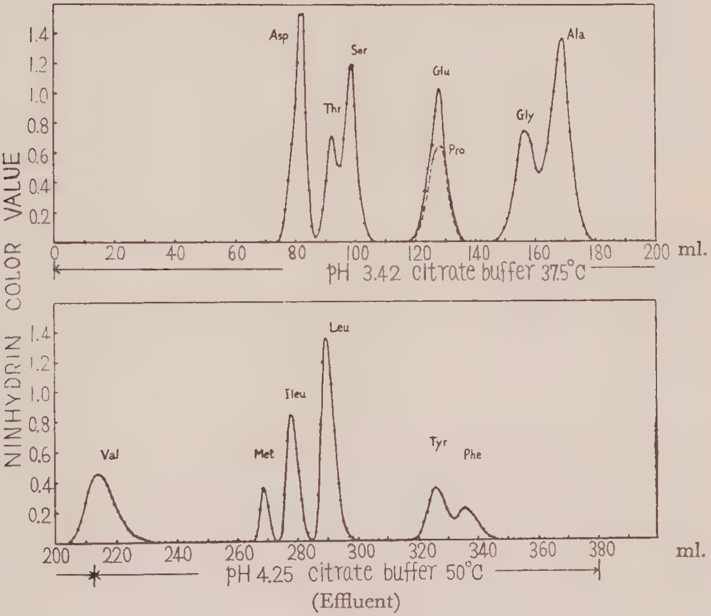
pH 6.8 phosphate buffer
pH 6.5 citrate buffer
C. Cysteic acid group: column 15×0.9 cm. Dowex-2
solvent; 0.1 *N* choloroacetic acid

The effluent was collected by Technicon's automatic fraction collector into 1.0 ml. portions.

Aportion of 2—3 mg. of the proteins were used for the chromatographic analysis. The chromatographic effluent patterns of phycoerythrin are shown in Figs. 2, 3 and 4 and the similar patterns were also obtained for phycocyanin.

Amino acid contents were calculated, from color index of each amino acid, taking that of leucine as 1.00 (*10*).

FIG. 2. Phycoerythrin.
(Dowex-50 0.9×100 cm)



RESULTS AND DISCUSSION

The results of analysis of amino acids are summarized in Table I, and II. The percentages of total nitrogen of the protein moieties calculated on the basis of the amino acid compositions were 81.35 per cent for phycoerythrin and 85.54 per cent for phycocyanin, respectively.

FIG. 3. Phycoerythrin
(Dowex-50 0.9×10 cm)

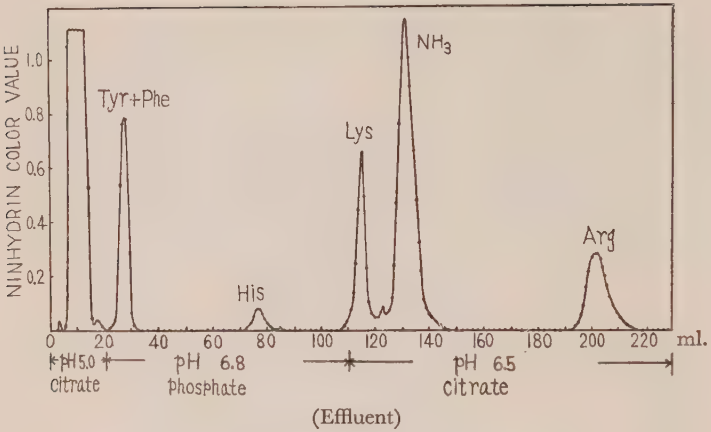
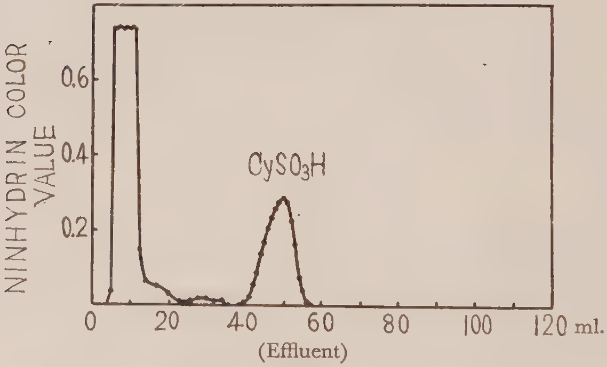


FIG. 4. Phycoerythrin
(Dowex 2 0.9×15 cm, 0.1 *N* Chloroacetic acid)



The residual nitrogen might have been as component of humin derived from the prosthetic groups.

The results of estimation made for 17 amino acids of the two chromoproteins indicate the close resemblance of their amino acid compositions except the remarkable difference in alanine, and slight differences in leucine, proline and tyrosine contents. Phycoerythrin contains more alanine and proline, but less leucine and tyrosine than phycocyanin.

TABLE I

Amino Acid Composition of Phycoerythrin

Amino acid	Amino acid in g./100 g. of protein	Amino acid residue in g./100 g. of protein	N in % of protein N	Amino acid in moles/10^5 g. protein
Aspartic acid	10.00	8.65	6.87	75.1
Glutamic acid	4.16	3.65	2.58	28.2
Serine	6.62	5.48	5.75	62.7
Threonine	2.84	2.41	2.19	23.8
Glycine	2.73	2.07	3.34	36.7
Alanine	12.50	9.97	12.80	140.0
Valine	5.80	4.91	4.55	49.5
Isoleucine	3.57	3.08	2.50	27.2
Leucine	5.66	4.87	3.96	43.1
Phenylalanine	1.62	1.44	0.90	9.9
Tyrosine	2.30	2.07	1.16	12.7
Proline	3.86	3.26	3.08	33.5
Histidine	0.70	0.62	1.24	4.5
Lysine	3.88	3.40	5.82	26.6
Arginine	6.03	5.41	12.70	34.8
Methionine	1.74	1.53	1.07	11.7
½ Cystine	0.85	0.73	0.65	7.1
			(4.59)*	
NH_3	1.88		10.18	
(Amide NH_3)**	(1.73)		(9.35)	
Totals................	77.75	63.55	81.35	627.1

* Calculated cystine value deduced methionin from total sulfur values.

** Amide NH_3 was determinated by ninhydrin technique of Hanafusa and Okada (*19*).

Both proteins have lower glutamic acid and phenylalanine contents in comparison with the other ordinary proteins and are especially low in glutamic acid as compared with vegetable proteins.

These proteins belong to pseudo-globulins according to Kyline (*13*), but the amino acid contents are low in leucine, isoleucine, glutamic acid and phenylalanine, and high in serine as compared with general globulins. As compared with hemoglobin, these proteins have lower content of histidine, lysine, glutamic acid and leucine, and higher of alanine, serine and arginine. Sisakyan, Rezingar and Kivktsan (*14*) have found qualitatively by paper chromatography two other components, α- and γ-aminobutyric acids in addition to the 17 amino acids of phycoerythrin cited above. Wassink and Ragetli (*15*) found 13 common amino acids and three unknown amino acids except

TABLE II

Amino Acid Composition of Phycocyanin

Amino acid	Amino acid in g./100 g. of protein	Amino acid residue in g./100 g. of protein	N in % of protein N	Amino acid in moles/10^5 g. protein
Aspartic acid	10.00	8.65	6.55	75.1
Glutamic acid	3.24	2.84	2.12	22.0
Serine	6.88	5.63	5.64	64.6
Threonine	3.61	3.07	2.65	31.5
Glycine	3.58	2.72	4.17	47.0
Alanine	8.98	7.16	8.80	100.1
Valine	5.18	4.38	3.87	44.2
Isoleucine	4.56	3.94	3.04	34.8
Leucine	8.43	7.28	5.61	64.3
Phenylalanine	2.76	2.46	1.46	16.7
Tyrosine	4.92	4.43	2.36	27.1
Proline	2.64	2.23	2.03	22.9
Histidine	0.21	0.19	0.33	1.4
Lysine	3.91	3.43	5.72	26.9
Arginine	7.31	6.55	13.60	41.8
Methionine	1.86	1.63	1.08	12.4
½ Cystine	0.95	0.81	0.70	7.9
			(2.09)*	
NH_3	2.20		11.40	
(Amide NH_3)**	(1.89)		(9.91)	
Totals............	.... 81.22	67.40	85.54	640.7

* Calculated cystine value deduced methionin from total sulfur values.

** Amide NH_3 was determinated by ninhydrin technique of Hanafusa and Okada (*19*).

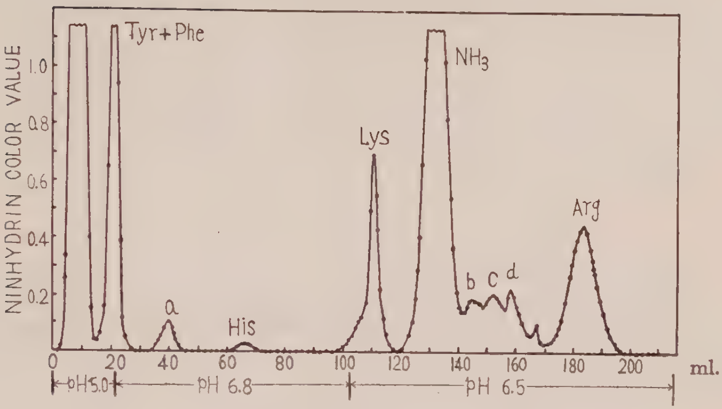
arginine in the phycocyanin of *Osillatoria.* The present author has also obtained four unknown peaks in chromatography of the hydrolysate of phycocyanin, three of them being observed in the basic fraction and another before histidine. The unknown peaks, as shown in Fig. 5, were observed at the same positions in repeated chromatographic experiments. They are probably new amino acids or peptides, but could not be identified in the present experiments.

Kitasato (*7*) reported the analytical results on phycoerythrin of Porphyra tenera as shown below.

amide-N	8.00	histidine-N	4.62
humin-N	3.02	lysine-N	9.63
cystine-N	4.94	monoamino-N	49.50
arginine-N	11.07	non protein-N	9.31

Total..............100.09

FIG. 5. Phycocyanin
(Dowex-50 0.9 × 15 cm)



a, b, c, d; unknown peaks (Effluent)

and they are somewhat different from the present results. Although Kitasato reported that phycoerythrin contained 1.33 per cent of tryptophan, the present author was not able to determine the tryptophan content by the disturbance of pyrrole cpmpounds derived from the prosthetic groups which could not be split completely away from their proteins by alkaline hydrolysis according to the method of Kitasato (*8*) and Lemberg (*6*). Moreover, the products split off by alkaline hydrolysis showed an absorption maximum at 550 mμ but did not show the charactertic absorption maximum of tryptophan at 610 mμ when treated with *p*-dimethylaminobenzaldehyde (*16*).

Amino acids containing sulfur were estimated as methionine and cysteic acid, the latter being derived from cystine and cysteine. No other amino acids containing sulfur could be detected by means of paper and ion exchange chromatograms. However, total amount of sulfur present as methionine and cysteic acid was lower than the total sulfur content. Theorell (*17*) and Tuppy *et al.* (*18*) reported that amino acid residues containing sulfur linked to the prothetic groups of cytochrome-c were not set free by acid hydrolysis, but remained combined with the pigments. Residual sulfurs might remain with prosthetic groups of phycoerythrin and phycocyanin, in the manner similer to that of thioether bridges of cytochrome c.

The contents of ammonia shown in Tables I and II are much higher than that calculated from aspartic and glutamic acid contents.

The author has, therefore, determinated amide N contents according to the ninhydrin technique of Hanafusa and Okada (*19*), after 3 hours hydrolysis with 2 *N* hydrochloric acid. The values obtained by this method are given in the tables. It must be assured that the chromoproteins contain some nitrogen compounds which easily release ammonia by acid hydrolysis. The results of this investigation indicated a high degree of similarity between the protein components of these two chromoproteins.

SUMMARY

Amino acid contents of crystalline phycoerythrin and phycocyanin from *Porphyra tenera* were determined by column-chromatographies using ion exchange resin Dowex-50 and Dowex-2. It was found that 17 common amino acids are present in these two chromoproteins and their amide *N* contents are very high.

The author wishes to express her gratefulness to Prof. S. Akabori for his interest and encouragement throughout this investigation and to Mr. H. Hanafusa for his valuable suggestions and technical help.

REFERENCES

(*1*) Haxo, F. T., and Bilnks, L. R., *J. Gen. Physiol.*, **6**, 391 (1950)
(*2*) French, C. S., and Young, V. K., *J. Gen. Physiol.*, **35**, 873 (1952)
(*3*) Bannister, T., *Arch. Biochim. Biophys.*, **49**, 222 (1954)
(*4*) Thomas, J. B., and Rover, W., *Biochem. Biophys. Acta*, **16**, 391 (1955)
(*5*) Fujiwara, T., *J. Biochem.*, **42**, 411 (1955)
(*6*) Lemberg, R., *Ann. Chem.*, **477**, 195 (1930)
(*7*) Lemberg, R., *Ann. Chem.*, **505**, 151 (1933)
(*8*) Kitasato, Z., *Act. Phytochim.* **2**, 274 (1925)
(*9*) Akabori, S., and Fujiwara, T., The 27th Congress of Japanese Biochemical Society, Kyoto April (1955)
(*10*) Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176**, 367 (1948)
(*11*) Moore, S. and Stein, W. H., *J. Biol. Chem.*, **192**, 663 (1951)
(*12*) Schram, E., Moore, S., and Bigwood, E. J., *Biochem. J.*, **57**, 33 (1954)
(*13*) Kyline, H., *Z. physiol. Chem.*, **60**, 169 (1910)
(*14*) Sisakyan, N. M., Bezinger, E. N., and Kivkutsan, F. R., *Doklady Akad. Nauk. S.S.S.R.*, **98**, 111 (1954)
(*15*) Wassink, E. C., and Ragetli H.W.J., *Pro. Koninkl. Ned. Akad. Wetenschap.* **55C**, 462 (1952)
(*16*) Spies, J. R., and Chambres, D. C., *Anal. Chem.*, **20**, 30 (1948); **21**, 1249 (1949)

(*17*) Theorell, H., *Biochem.*, *Z.*, **298**, 242 (1938)
(*18*) Tuppy, H., and Paleus, S., *Acta Chem. Scand.*, **9**, 353 (1955)
(*19*) Okada, Y., and Hanafusa, H., *Bull. Chem. Soc. Japan*, **27**, 478 (1954)

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ACCEPTOR RELATIONS IN THE ENZYMATIC TRANSFER OF β-GLUCOSYL AND β-GALACTOSYL GROUPS*

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(Received for publication, January 16, 1956)

In an earlier communication (*1*), it has been shown that the β-glucosyl group of aryl-β-glucoside is enzymatically transferred to alcohol with the formation of alkyl-β-glucoside. At that time the transferring reaction was found to take place solely with the dry powder of green leaves, as in the case of Rabaté (*2*) who discovered this reaction, but later the reaction was widely recognized in ordinary β-glucosidase preparations, such as those from molds or apricot emulsin (*3*). Contrary to the view of Rabaté we were of the opinion that the transferring reaction of β-glucosyl was catalyzed by β-glucosidase itself, not by a particular transferring enzyme (*3*).

Subsequently, the enzymatic transfer of β-galactosyl group of aryl-β-galactoside to alcohol has also been demonstrated with β-galactosidase of apricot emulsin and green leaves (*4*).

The hydrolysis and the transfer action of glycosidase have been interpreted in terms of acceptor differences: the hydrolysis occurs by the transfer of the glycosyl groups to water molecule, while the transfer reaction takes place when these groups are transferred to appropriate acceptor molecules (*3, 4*). It may be presumed that the acceptor specificity which is generally recognized in enzymatic dehydrogenations might also exist in the transglycosylation. So long as the transglycosylation reactions are considered to be intimately related to the biosynthesis of saccharides, the clarification of the acceptor specificity seems important in that it determines the nature of the transfer products.

In the present study research has been made about the acceptor specificity in the transglycosylation reaction of β-glucosidase and β-galactosidase from apricot emulsin and leaves of higher plants.

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* A part of this study was reported before the 8th. Symposium on Enzyme Chemistry held in July, 1955 in Osaka.

EXPERIMENTAL

Substrates and Acceptors—*p*-Nitrophenyl-β-D-glucoside and *p*-nitrophenyl-β-D-galactoside were used as substrates (Donor). As acceptors were used following hydroxy compounds: methanol, ethanol, *n*-propanol, isopropanol, *n*-butanol, isobutanol, isoamylalcohol, isooctanol, benzylalcohol, allylalcohol, ethylene glycol, glycerol, erythritol, mannitol, *meso*-inositol, potassium malate and potassium lactate.

Enzyme Preparations—Enzyme solution of elder leaves (*Sambucus Sieboldiana*) was prepared by the procedure described previously (*4*). Apricot emulsin was prepared according to the procedure of Helferich *et al.* for the preparation of almond emulsin (*5*). Its specific activity nearly corresponded to that of " Rohferment " of Helferich.

Assay—The hydrolytic and transfer activities of the enzyme were estimated as follows: hydrolase activity was measured by the estimation of the liberated *p*-nitrophenol and the reducing sugars in the absence of alcohol and the transferase activity in its presence. For the quantitative determination of *p*-nitrophenol, 0.5 ml. aliquots were withdrawn from the reaction mixture (10 ml.) and diluted to 20 ml. with water. To 1.0 ml. of the diluted solution 4 ml. of 10 per cent sodium carbonate were added and the intensity of the color developed was estimated colorimetrically.

The estimation of the liberated glucose or galactose was carried out reductometrically with 1.0 ml.- aliquots by Shaffer-Hartman-Somogyi titration. The extent of glycosyl transfer was expressed by the following formula:

$$\frac{[p\text{-Nitrophenol liberated}]-[\text{sugar liberated}]}{[p\text{-Nitrophenol liberated}]}\times 100$$

RESULTS

Acceptor Relations in the Enzymatic Transfer of β-Glucosyl Group

With various hydroxy compounds as acceptors for β-glucosyl group the transfer activity of the β-glucosidase preparations from two different sources was estimated. From the results shown in Tables I and II it will be seen that, with few exceptions, almost all these compounds could serve as acceptor for the β-glucosyl group. Comparison of Tables I and II reveals that there exist both qualitative and quantitative differences in the use of the acceptor between the β-glucosidase of apricot emulsin and that of the elder leaves. Thus, for example, *n*-butanol represents an efficient acceptor with apricot enzyme while it is a relatively weak one with elder preparation. Also no appreciable transfer of β-glucosyl to isoamylalcohol, erythritol and mannitol was observed with apricot emulsin whereas remarkable transfer to these alcohols took place with the preparation of elder leaves. Under the present conditions, isooctylalcohol is unable to act as acceptor. This would be partly due to its slight solubility in water. With the elder enzyme the cleavage of *p*-nitrophenyl-β-D-glucoside is accelerated in the presence of *n*-propanol, ethylene glycol, glycerol and allylalcohol, as revealed by comparing the values with and without alcohol.

Influence of Alcohol Concentration

If water and alcohol act respectively as acceptor for sugar residue, the competition would be expected to occur between alcohol and water for the specific surface of the enzyme. Thus the rise in the alcohol concentration might result in the increased extent of the glucosyl transfer.

TABLE I

Enzymatic Transfer of β-Glucosyl from p-Nitrophenyl-β-D-glucoside to Various Alcohols by the β-Glucosidase of Sambucus Sieboldiana (Elder)

Reaction mixture (final concentration): substrate (*p*-nitrophenyl-β-D-glucoside), 0.0096 *M*; acetate buffer, pH 4.8, 0.04 *M*; enzyme solution, 4 ml.; acceptor*, 1.0 *M*. Total volume, 10 ml.; Temp. 30°. As control water was used in place of alcohol.

(* In the case of alcohols of low solubility saturated aqueous solution was used.)

A

Acceptor (—alcohol)	Incubation time	*p*-Nitrophenol liberated	Glucose liberated	Glucose transferred to alcohol
	min.	%	%	%
Control (water)	60	15	17.0	
	120	30	33.7	
	180	45	47.0	
	300	65	64.5	
n-Propyl-	120	55	19.9	64
	180	65	22.8	65
	300	75	28.5	62
Isopropyl-	120	30	17.0	43
	180	45	27.6	39
	300	60	32.2	46
n-Butyl-	120	38	10.2	73
	300	75	23.6	69
Isobutyl-	120	40	26.6	34
	180	48	32.2	33
	300	60	39.7	35
*Isoamyl-	120	28	21.4	24
	180	45	25.5	38
	300	55	30.6	33
*Isooctyl-	120	25	23.6	6
	180	28	30.0	−7
	300	38	41.0	−8

B

Control (water)	60	20	22.9	
	120	32	33.0	
	180	45	49.3	
	300	65	67.0	
Benzylalcohol	120	27	20.6	24
	180	35	27.4	22
	300	55	43.0	22
Ethylene glycol	120	45	22.8	49
	180	65	30.8	53
	300	80	36.6	54
Glycerol	60	23	11.1	52
	120	45	20.6	54
	180	60	22.8	62
	300	80	25.1	69
Mannitol	120	35	26.6	24
	180	48	30.0	38
	300	68	35.9	47
Lactate	180	27	27.6	–2
	300	35	36.6	–5
	400	50	51.1	–2

C

Control (water)	90	30	30.4	
	150	40	41.2	
	210	53	51.0	
meso-Inositol	90	30	22.8	24
	150	40	28.5	29
	210	53	38.5	27
Erythritol	90	35	17.0	51
	150	45	23.6	48
	210	60	30.0	50
Allylalcohol	90	45	18.4	59
	150	65	24.3	62
	210	75	28.5	62
Malate	90	18	20.6	–14
	150	25	25.0	0
	210	30	32.2	–7
	420	50	51.0	–2

So the influence of alcohol concentration on glucosyl transfer was examined with methanol, ethanol and *n*-propanol. Table III indicates the results of the experiment with apricot emulsin.

From the figures in Table III it is evident that the extent of glucosyl transfer from *p*-nitrophenyl-β-D-glucoside to methanol, ethanol or *n*-propanol increased with the increasing concentration of alcohol. However, as might be expected, higher concentration of the alcohol was more or less inhibitory.

It is well known that as a result of the reverse reaction of the hydrolysis β-glucosides are formed on incubating a highly concentrated mixture

TABLE II

Enzymatic Transfer of β-Glucosyl from p-Nitrophenyl-β-D-glucoside to Various Alcohols by the β-Glucosidase of Apricot Emulsin

Conditions of reaction, as described in Table I.

A

Acceptor (-alcohol)	Incubation time	*p*-Nitrophenol liberated	Glucose liberated	Glucose transferred to alcohol
	min.	%	%	%
Control (water)	15	60	56.0	
	30	85	86.1	
	240	93	91.4	
n-Propyl-	15	50	26.6	47
	30	70	42.1	40
	240	90	52.1	41
Isopropyl-	15	52	38.9	25
	30	85	57.5	32
	240	93	71.4	23

B

Control (water)	20	45	43.0	
	40	65	63.0	
	60	80	82.5	
n-Butyl	20	20	14.3	29
	40	38	28.5	25
	60	62	43.0	31
Benzyl-	20	25	11.0	56
	40	34	20.6	39
	60	40	24.3	39
Allyl-	20	55	39.6	28
	40	70	51.0	27
	60	80	54.8	32
Glycerol	20	30	19.9	34
	40	55	27.5	50
	60	74	38.2	48
Ethylene glycol	20	40	19.9	50
	40	65	35.2	46
	60	80	40.2	50
Erythritol	20	18	17.1	5
	40	32	30.8	4
	60	42	39.8	5
meso-Inositol	20	32	27.6	14
	40	55	42.0	24
	60	70	55.2	21
Mannitol	20	35	—	—
	40	55	55.9	-2
	60	70	71.4	-2

C

Control (water)	20	32	33.0	
	40	52	52.7	
	60	60	63.0	
*Isoamylalcohol	20	18	18.4	–2
	40	35	34.5	1
	60	45	46.9	–4
*Isooctylalcohol	20	32	32.2	–0.6
	40	45	46.0	–2
	60	58	56.8	2

D

Control (water)	20	42	43.8	
	40	60	59.1	
	60	75	75.0	
Lactate	20	42	43.8	–3
	40	60	62.2	–4
	60	73	72.2	1
Malate	20	35	36.6	–5
	40	55	55.1	–0.8
	60	65	62.9	3

TABLE III

Relation between Extent of β-Glucosyl Transfer and Concentration of Alcohol with Apricot Emulsin

Reaction mixture (final concentration): substrate (*p*-nitrophenol-β-D-glucoside), 0.0096 *M*; acetate buffer, pH 4.8, 0.04 *M*; enzyme solution, 4 ml.; concentration of alcohols as indicated. Total volume, 10 ml; Temp. 30°.

A

Final concentration of methanol	Incubation time	*p*-Nitrophenol liberated	Glucose liberated	Glucose transferred to alcohol
M	*min.*	%	%	%
Control (water)	30	45	46.0	
	75	70	72.3	
	120	85	87.6	
1.23	30	42	30.0	28
	75	65	47.5	27
	120	83	60.7	27
2.46	75	60	31.5	48
	120	80	36.6	54
	240	90	41.2	54
4.92	75	52	16.1	69
	120	70	19.1	73
	240	90	30.0	67
7.35	30	20	4.9	75
	75	40	10.0	75
	120	60	11.0	81
	240	78	13.2	83

B

Final concentration of propanol	Incubation time	*p*-Nitrophenol liberated	Glucose liberated	Glucose transferred to alcohol
M	*min.*	%	%	%
Control (water)	50	38	37.4	
	120	55	55.0	
	240	65	66.0	
0.69	50	22	14.3	35
	120	35	24.3	31
	240	50	31.5	37
1.34	50	18	10.0	45
	120	47	26.6	43
	240	75	39.6	47
2.68	50	18	9.1	47
	240	37	19.2	48
	1500	72	34.5	52
4.02	240	20	9.1	55
	1500	48	17.0	65

C

Final concentration of ethanol	Incubation time	*p*-Nitrophenol liberated	Glucose liberated	Glucose transferred to alcohol
M	*min.*	%	%	%
Control (water)	70	30	30.8	
	180	50	51.0	
	360	65	61.3	
0.84	70	28	19.2	31
	180	48	32.4	32
	360	65	42.0	30
1.69	70	25	13.2	47
	180	38	22.1	42
	360	60	30.7	44
3.38	70	15	6.1	59
	360	40	19.2	52
	660	65	26.6	59
5.07	360	40	13.2	67
	660	50	19.9	62

of glucose and alcohol with emulsin (*9*). In view of the fact that some of the above transfer reactions were carried out under relatively high concentration of the alcohol, it seemed worth while to examine whether or not such reverse reactions might have been responsible for the formation of alkyl-β-glucosides. However, under experimental conditions employed no reversal of the hydrolysis could be observed, presumably due to the low concentration of glucose (Table IV).

TABLE IV

Action of Apricot Emulsin on Glucose and Methanol

Final concentration: D-glucose, 0.0096 *M*; acetate buffer, pH 4.8, 0.04 *M*; methanol, 7.38 *M*; 50 mg emulsin. Total volume, 10 ml.

Incubation time	Free glucose
min.	*mg./10 ml.*
0	18.3
180	18.4
270	18.1
600	18.5

Acceptor Relations in the Enzymatic Transfer of Galactosyl Group

Enzymatic transfer of β-galactosyl from aryl-β-galactoside to methanol or ethanol was reported in a previous paper (*4*). In the present work, it was investigated whether alcohols other than methanol and ethanol would act as acceptor of galactosyl group. As shown in Tables V and VI, most of these alcohols served as the acceptor of the enzymatically liberated β-galactosyl group. As with the β-glucosyl transfer isooctylalcohol was inactive with the β-galactosidases of both apricot emulsin and elder leaves while isoamylalcohol was able to act as an acceptor only with the enzyme of elder leaves.

DISCUSSION

In earlier communications we have shown that glycosidases which had been considered as hydrolyzing enzymes are essentially nothing but transglycosylases where water molecule represents like alcohols as an acceptor for glycosyl groups (*1*, *3*, *4*). Similar view has been shared by Bacon and his coworkers (*6*). From this point of view it was considered probable that the acceptor specificity which has generally been recognized with dehydrogenases might equally be found in transglycosylases. The present study showed that this was really the case.

Of the results summarized in Table VII following points may be worth mentioning.

i) The availability of alcohols in the enzymatic sugar transfer as expressed by the "extent of transglycosylation" varies more or less significantly with the type of alcohols even when one and the same enzyme preparation of β-glucosidase or β-galactosidase was employed.

TABLE V

Enzymatic Transfer of β-Galactosyl Group from p-Nitrophenyl-β-D-galactoside to Various Alcohols by β-Galactosidase of Elder Leaves

Reaction mixture (final concentration): substrate (*p*-nitrophenyl-β-D-galactoside), 0.0096 *M*; acetate buffer, pH 4.8, 0.04 *M*; enzyme solution, 4 ml.; acceptor, 1.0 *M*; Total volume, 10 ml.; Temp. 30°.

As control water was used in place of alcohol.

Acceptor (–alcohol)	Incubation time	*p*-Nitrophenol liberated	Galactose liberated	Galactose transferred to alcohol
	min.	%	%	%
Control (water)	90	20	20.3	
	180	30	31.5	
	270	40	40.2	
	360	50	49.8	
n-Propyl-	180	40	13.9	65
	270	50	16.6	67
	360	60	19.1	71
Isopropyl-	180	40	22.3	44
	270	60	26.0	57
	360	70	31.5	55
n-Butyl-	90	18	10.3	43
	180	27	12.5	54
	270	32	13.9	57
Isobutyl-	90	22	11.4	48
	180	30	13.9	49
	270	35	16.6	53
	600	42	20.6	51
*Isoamyl-	90	20	11.4	43
	180	25	13.9	44
	270	32	16.6	52
	600	35	19.1	45
*Isooctyl-	180	20	20.3	–2
	600	30	31.5	–5

ii) Whichever alcohols are more efficient as the acceptor of transferred sugar residue varies with the origin of the enzyme preparations either of β-glucosidase or of β-galactosidase.

Analogous phenomena have been observed by Bacon and Bealing (*6*) with sucrase (β-fructofuranosidase). When yeast sucrase was used benzylalcohol was found to serve as an acceptor for β-fructofuranosyl group whereas with mold enzyme this alcohol failed to do so. Kurasawa *et al.* (*7*) reported the existence in the strains of *Aspergillus oryzae* of two distinct types of sucrases differring each other in the acceptor specificity. The one is characterized by the ability to react besides with water also with sugars as acceptor forming oligosaccharides while

TABLE VI

Enzymatic Transfer of β-Galactosyl Group from p-Nitrophenyl-β-D-galactoside to Various Alcohols by β-Galactosidase of Apricot Emulsin

Conditions of reactions, as described in Table V.

A

Acceptor (-alcohol)	Incubation time	*p*-Nitrophenol liberated	Galactose liberated	Galactose transferred to alcohol
	min.	%	%	%
Control (water)	25	30	28.5	
	85	50	53.8	
	345	90	92.0	
n-Propyl-	90	40	19.0	52
	1450	93	46.0	51
Isopropyl-	85	55	46.0	19
	345	92	74.2	19
n-Butyl-	85	28	10.2	64
	345	65	24.8	62
Isobuty-	85	30	15.2	49
	345	65	38.2	41

B

Control (water)	100	60	60.4	
	200	70	74.8	
*Isoamylalcohol	100	15	15.2	−1
	200	20	23.2	−16
*Isooctylalcohol	100	30	31.4	−4
	200	40	44.0	−10

the other reacts preferentially with water and lower alcohols such as methanol or ethanol.

In their study on the glucosidase specificity Miwa and coworkers (*8*) showed that the effect of the chemical structur of aglucones upon the rate of the enzymatic hydrolysis of β-glucosides varied considerably with the origin of the enzyme preparation. These results have been taken to indicate the existence of different β-glucosidases of varying aglucone specificity. Such a multiplicity in the aglycone specificity of the glycosidase seems to be reflected on the acceptor specificity in transglycosylation.

In the present experiment a tendency was noticed in both β-glucosidase and β-galactosidase that the enzyme preparations from elder leaves displayed relatively higher degree of transfer activity than apricot emulsin. Whether or not any physiological significance could be ascribed to the presence in leaves of glycosidases of high transfer activity

TABLE VII

Transfer of Glycosyl Groups to Various Acceptors by the Enzyme of Elder Leaves and Apricot Emulsin

Acceptor (-alcohol)	β-Glucosyl group transferred to acceptor by enzyme of		β-Galactosyl group transferred to acceptor by enzyme of	
	Elder leaves	Apricot emulsin	Elder leaves	Apricot emulsin
	%	%	%	%
Methyl-	32–46	27–29	55–61	14–19
Ethyl-	32–46	30–38	54–63	14–21
n-Propyl-	62–65	40–47	65–71	51–52
Isopropyl-	39–46	23–32	44–57	19
n-Butyl-	68–73	25–31	43–57	62–64
Isobutyl-	33–35	——	48–53	41–49
*Isoamyl-	22–37	0	43–52	0
*Isooctyl-	0	0	0	0
Benzyl-	21–24	39		
Ethylene glycol	49–54	46–50		
Glycerol	52–69	34–50		
Mannitol	24–47	0		
meso-Inositol	24–29	14–24		
Erythritol	48–52	0– 5		
Allyl-	59–63	27–32		
Lactate	0	0		
Malate	0	0		

remains as yet obscure.

SUMMARY

1. In extending our previous studies a number of hydroxy compounds were tested for their acceptor function in the enzymatic transglycosylation using β-glucosidase and β-galactosidase preparations from elder (*Sambucus Sieboldiana*) leaves and apricot emulsin.

2. It was found that most of aliphatic alcohols were able to act as the acceptor of β-glucosyl and β-galactosyl groups, while lactate and malate were inactive in this reaction.

3. In respect of either β-glucosidase or β-galactosidase the efficiency

of various alcohols as acceptor in the transglycosylation differred between the enzyme of elder leaves and that of apricot emulsin, indicating that the acceptor specificity of the enzyme catalyzing transglycosylation may differ with the source of the enzyme.

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REFERENCES

(*1*) Miwa, T., Takano, K., Mafune, K., and Furutani, S., *Proc. Japan Acad.*, **25**, 111 (1949)

(*2*) Rabaté, J., *Bull. Soc. Chim. biol.*, **17**, 572 (1935); **20**, 449 (1938)

(*3*) Takano, K., and Miwa, T., *J. Biochem.*, **37**, 435 (1950)

(*4*) Takano, K., and Miwa, T., *J. Biochem.*, **40**, 471 (1953)

(*5*) Helferich, B., Winkler, S., Gootz, R., Peters, O., and Günther, E., *Z. physiol. Chem.*, **208**, 91 (1932)

(*6*) Bacon, J.S.D., *Biochem. J.*, 50, xviii (1952); Bealing, F. J., *Biochem. J.*, **55**, 93 (1953)

(*7*) Kurasawa, H., Saito, S., Homma, N., and Yamamoto, Y., *Bull. Fac. Agr. Niigata Univ.*, **5**, 50 (1954); **7**, 57 (1955)

(*8*) Miwa, T., Cheng, C., Fujisaki, M., and Toishi, A., *Acta. Phytochim.*, **10**, 155 (1937); *Sci. Rep. Tokyo Bunrika Daigaku*, B, **6**, 11 (1942)

(*9*) Bourquelot, E., *Ann. chim.*, **29**, 145 (1913)

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AMINO ACID COMPOSITION OF RIBONUCLEOPROTEIN OF SILKGLAND*

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In the study of biosynthesis of protein, one of the essential problems with which we are confronted is to account for the high degree of specificity of the protein formed. This specificity is considered to be attributed to a specific chemical and physical structure of protein. Although it has been demonstrated that nucleic acid or nucleoprotein, especially ribonucleic acid or ribonucleoprotein, plays an important and indeed fundamental part in the synthesis of protein, the exact nature of the mechanism by which they act is more difficult to determine. Haurowitz (*1*) proposed an idea that the synthesis of protein takes place as a reproduction of positive replica of the template. In his assumption the template is considered to be the protein combined with nucleic acid. Recently Dounce (*2*) has suggested that the template governing the order of arrangement of amino acids in peptide chain is polynucleic acid itself. Similar explanation was described by Borsook (*3*). However, Campbell and Work (*4*) have reviewed on the template theory with a critical eye.

The purpose of the present experiments was to inspect the role of ribonucleoprotein as a template in protein synthesis. The ribonucleoprotein was prepared from the posterior silkgland of silkworm and its amino acid composition was compared with that of silk fibroin, which is well known to be synthesized in the posterior gland. If the protein moiety of ribonucleoprotein do act as a template for the synthesis of fibroin molecule, a similarity in amino acid composition between the ribonucleoprotein of silkgland and silk fibroin would be expected.

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* A preliminary report of this work was presented at the 27th Meeting of Japanese Biochemical Society held in Sendai, in April 1954.

EXPERIMENTAL

Isolation and Purification of the Ribonucleoprotein—Griffin *et al.* (*5*) have isolated the ribo- and desoxyribo-nucleoprotein of rat liver, extracting with 0.4 *M* NaCl and 1 *M* NaCl, respectively. In the present investigation the ribonucleoprotein was isolated from the posterior silkgland by a slight modification of Griffin's procedure.

Silkworms (*Bombyx mori*, L.; a hybrid "Japanese 112×Chinese 112") in the 5th day of the 5th instar, were dissected in a 0.14 *M* NaCl. The posterior silkglands were excised and washed three times with ice-cold 0.14 *M* NaCl. To the wet silkgland tissue were added 3.5 parts by weight of neutral 0.4 *M* NaCl and the mixture was homogenized for 5 minutes in a glass homogenizer at 0°. The homogenate was centrifuged for 15 minutes at 4000 r.p.m. The supernatant fluid was removed and the residue again extracted with a volume of 0.4 *M* NaCl equal to that of the above supernatant fluid. The combined solution of the first and second extracts with 0.4 *M* NaCl was acidified with 0.1 *N* HCl to pH 4.2, allowed to stand for 1 hour, and was then centrifuged for 15 minutes at 4000 r.p.m. The supernatant solution was discarded. The precipitate was suspended in 50 ml. ice-cold distilled water and 0.1 *N* NaOH was added slowly to dissolved the ribonucleoprotein with a strong stirring. The final pH was adjusted to 8.0. A considerable amount of protein was remained undissolved, which was centrifuged off, and the supernatant was again reduced to pH 4.2 with 0.1 *N* HCl. The precipitate of ribonucleoprotein appeared immediately. The same procedure of precipitation was repeated more three times. In the course of reprecipitation each precipitate was analyzed for total nitrogen and phosphorus. In a preliminary test it was found that the N:P ratio did not increase by the further precipitation. At the end of fifth precipitation the ribonucleoprotein was washed with cold water, alcohol, and ether as usual. 530 mg. of purified ribonucleoprotein was obtained from 100 g. posterior silkgland.

Determination of Amino Acid—Either chemical or chromatographical method was used for the determination of amino acid. The ribonucleoprotein to be analyzed was hydrolyzed with 1:1 HCl for twenty hours at 110°. Glycine was chemically determined by the procedure of Krueger (*6*), serine by the procedure of O'Dea (*7*), threonine by the procedure of Winnick (*8*) and Neidig (*9*) with slight modifications, tyrosine by the procedure of Folin (*10*), and arginine by the procedure of Sakaguchi (*11*). Glutamic acid, aspartic acid, alanine, leucine, valine, and proline were determined by a quantitative column- and paper-chromatography of Maruta *et al.* (*12*).

Purified silk fibroin was also analyzed for the amino acids described above for the sake of comparison with the ribonucleoprotein.

Identification of N-Terminal Amino Acids—The *N*-terminal amino acids of the ribonucleoprotein were identified by dinitrofluorobenzene method. The procedure was essentially that of Sanger (*13*). After treating 100 mg. of the ribonucleoprotein with dinitrofluorobenzene, the DNP-ribonucleoprotein was hydrolyzed in 1:1 HCl at 105° to 110° for 8 hours and the DNP-amino acids were extracted with ether. The ether extract and the aqueous layer were condensed in vacuo, and then subjected to paper electrophoresis and followed by paper chromatography as usual.

RESULTS

Purity of the Ribonucleoprotein—The contents of nitrogen and phosphorus of the isolated ribonucleoprotein were as follows:

$$\text{N } 147\,\gamma/\text{mg.},\quad \text{P } 28.5\,\gamma/\text{mg.},\quad \text{N:P ratio}=5.2$$

Ribonucleic acid phosphorus was also determined by ultraviolet absorption at 260 mμ in a Beckman spectrophotometer according to Ogur and Rosen (*14*) and found to be 25.3 γ per mg. ribonucleoprotein. As the average phosphorus content of ribonucleic acids is 9.4 per cent (*15*), so the ribonucleic acid moiety of the ribonucleoprotein could be calculated as 26.8 per cent ($2.53\times100/9.4$).

Electrophorectic study was also carried out by using 0.05 *M* phosphate buffer of 0.144 ionic strength at pH 8.0. The pattern was run at 100 V. and 1.5 mA. at 13°. The ascending and desending patterns show one colloidal boundary, which indicates that the nucleoprotein moves as one component at pH 8.0. Owing to the limited amount of purified ribonucleoprotein, it was impossible to carry out further electrophoretic studies.

Amino Acid Composition—In Table I the amino acid compositions of the riboucleoprotein and silk fibroin are shown. The data are stated in terms of weights of amino acids per 100 g. of protein.

The most remarkable difference between these two proteins can be seen in their contents of glycine and alanine, the high content of which is generally considered to be one of most characteristic properties of silk fibroin. Moreover, it should be point out that the ribonucleoprotein contains considerably larger amounts of glutamic acid and aspartic acid than fibroin. These results appear to indicate little similarity between the amino acid compositions of the two proteins.

N-Terminal Amino Acid—The ether extract from the acid hydrolysate of DNP-ribonucleoprotein was submitted to paper-electrophoretic fractionation (0.1 *M* phosphate buffer of pH 7.8, 350 V.). It is separated into four principal bands, I, II, III, and IV, as shown in Fig. 1. Each band was eluted with 4 per cent sodium bicarbonate solution and the eluates were further subjected to one dimentional paper chromatography using the solvent system butanol-ammonia or benzylalcohol-ethanol (9:1).

Spot I was identified as dinitrophenol.

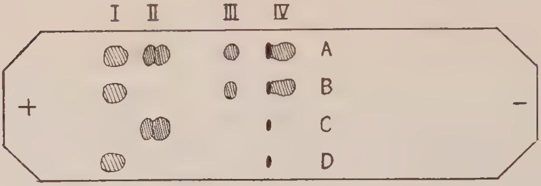

FIG. 1. Paper electrophoresis of *N*-terminal DNP-amino acids of DNP-ribonucleoprotein.

A: DNP-ribonucleoprotein of silkgland.
B: DNP-ribonucleic acid of yeast.
C: DNP-glycine+DNP-alanine+DNP-Serine.
D: 2,4-Dinitrophenol.
(0.1 *M* phosphate buffer of pH 7.8, 350 V., 3 hours.)

Spot II separated into three yellow spots which were identified as DNP-derivatives of glycine, alanine and serine, and also checked by hydrolysing the compounds with ammonia in a sealed tube (*16*).

Each band of III and IV could not be effectively resolved by paper chromatography and they showed somewhat different behaviors from known DNP-amino acids. They were, therefore, compared with the ether extract from the acid hydrolysate of DNP-ribonucleic acid* (yeast) on paper-electrograms. The similar spots corresponding band III and IV were obtained in the case of DNP-ribonucleic acid. The eluates of these spots gave a typical ultraviolet spectrum of nucleic acid, having absorption maxima near 262 mμ and minima near 245 mμ. From these observations the two spots appear to be degradative products of DNP-nucleic acid.

The same analytical procedures mentioned above were applied on DNP-fibroin to determine the *N*-terminal residues and glycine, alanie and serine could be detected. This is in agreement with the results obtained by Narita (*17*).

DISCUSSION

On the mechanism of protein synthesis, it has been generally assumed that the specificity of protein formed is determined by a template. As to the nature of this template, Haurowitz (*1*) has proposed a hypothesis that a template for the formation of a monomolecular protein film consists of a protein part of nucleoprotein and the role of the nucleic acid is to remain the template protein film in the expanded state. Dounce (*2*), on the other hand, suggested that ribonucleic acid may act as templates for protein synthesis.

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If the former mechanism has a great role in the synthesis of silk fibroin in posterior gland of silkworm, it may be expected that there is some similarity in amino acid composition, especially in respect of the contents of glycine and alanine, between the ribonucleoprotein isolated from silkgland and the fibroin synthesized in the gland tissue. The result given in Table I, however, shows little similarity in amino acid composition of the two proteins. It seems unlikely, therefore, that the protein moiety of ribonucleoprotein can act as a template in the process of protein synthesis.

However, the data concerning the *N*-terminal amino acid of the

TABLE I

Amino Acid Composition of Ribonucleoprotein Isolated from Silkgland

Amino acid	Ribonucleoprotein		Silk fibroin	Method of determination
	Per cent per 100 g. ribonucleoprotein	Per cent per 100 g. protein moiety		
Glycine	2.5	3.4	38.0	C
Alanine	5.0	6.8	24.2	P
Serine	9.9	13.5	16.4	C
Tyrosine	3.1	4.2	9.6	C
Valine	5.7	7.8	3.4	P
Aspartic acid	9.8	13.4	3.3	P
Glutamic acid	9.9	13.5	2.3	P
Phenylalanine	2.6	3.6	1.4	C
Threonine	2.0	2.7	1.1	C
Leucine, isoleucine	7.3	10.0	2.2	P
Proline	1.3	1.8	0.24	P
Arginine	7.1	9.7	0.75	C
Total	——	90.4	103.0	

C—Chemical method
P—Quantitative paper chromatographic method

two proteins seem to suggest the presence of some correlations between the two proteins. Thus it appears impossible to deny all relationship in respect of amino acid arrangement. Although the amino acid compositions of the two proteins show little similarity, a possibility remains that a part of the ribonucleoprotein molecule may have amino acid sequences similar to that of fibroin molecule and such a part can act as a template in protein synthesis.

SUMMARY

1. Ribonucleoprotein was isolated from the silkgland of silkworm (*Bombyx mori*, L.) in the 5th day of the 5th instar.

2. The amino acid composition of the ribonucleoprotein was analyzed by chemical or quantitative paperchromatographical methods, and compared with that of silk fibroin.

3. Little similarity between the amino acid compositions of the two proteins was observed.

4. *N*-Terminal amino acid residues of the ribonucleoprotein were shown qualitatively to be occupied by glycine, alanine and serine, and this is similar to fibroin.

REFERENCES

(*1*) Haurowitz, F., Chemistry and biology of proteins, Academic Press, New York, p. 326 (1951)
(*2*) Dounce, A. L., *Enzymologia*, **15**, 251 (1953)
(*3*) Borsook, H., *3rd Intern. Congr. Biochem.*, p. 54, Brussel, Belgium (1955)
(*4*) Campbell, P. N., and Work, T. S., *Nature*, **172**, 541 (1953)
(*5*) Griffin, A. C., Nye, W. N., Noda, L., and Luck, J. M., *J. Biol. Chem.*, **176**, 1225 (1948)
(*6*) Krueger, R., *Helv. Chim. Acta*, **32**, 238 (1949)
(*7*) O'Dea, J. F., and Gibbone, R. A., *Biochem. J.*, **55**, 580 (1953)
(*8*) Winnick, T., *J. Biol. Chem.*, **142**, 461 (1942)
(*9*) Neidig, B. A., and Hess, W. C., *Anal. Chem.*, **24**, 1627 (1952)
(*10*) Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927)
(*11*) Sakaguchi, S., *J. Biochem.*, **37**, 231 (1950)
(*12*) Maruta, Y., Samejima, H., and Tanaka, M., *J. Agr. Chem. Soc. Japan*, **28**, 775 (1954)
(*13*) Sanger, F., *Biochem. J.*, **39**, 507 (1945); *ibid.*, **45**, 563 (1949)
(*14*) Ogur, M., and Rosen, G., *Arch. Biochem.*, **25**, 262 (1950)
(*15*) Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 83 (1945)
(*16*) Lowther, A. G., *Nature*, **167**, 767 (1951)
(*17*) Narita, K., *J. Chem. Soc. Japan.*, **75**, 1005 (1954)

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NITRATE REDUCTION IN AEROBIC BACTERIA AND THAT IN ESCHERICHIA COLI COUPLED TO PHOSPHORYLATION

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It is well known that some facultative anaerobic bacteria can grow rapidly under anaerobic conditions in a medium containing nitrate. This observation suggests that nitrate is used as an oxidizing agent in place of molecular oxygen (*1*, *2*), in other words, that "nitrate respiration" (*3*) is occurring. In the present study, the nitrate respiration of some strictly aerobic bacteria was examined. Several strains of aerobic bacteria showed typical nitrate respiration, whereas some strains showed negligible growth in a nitrate containing medium under anaerobic conditions. The latter group of bacteria, however, displayed nitrate reduction when grown aerobically in the same medium. These observations might be explained by the multifunctional nature of the nitrate reducing system as has been discussed by Taniguchi, Sato, and Egami. (*4*).

It is widely accepted that when oxygen is used as an ultimate electron acceptor, the energy liberated by the oxidation is pooled as phosphate bond energy. Since some aerobic and facultative anaerobic bacteria showed rapid growth in the presence of nitrate under anaerobic conditions, it is reasonable to assume that phosphorylation is occurring coupled to the reduction of nitrate. In confirmation of this view, the incorporation of P^{32}-labelled orthophosphate into *Escherichia coli* cells was greatly enhaced by nitrate reduction. The data, still preliminary and indirect, support a coupling of phosphorylation to nitrate respiration.

METHODS AND MATERIALS

Microorganisms—The following aerobic bacteria were kindly supplied from the Culture Stock Division of the Institute of Applied Microbiology, University of Tokyo: *Bacillus subtilis* P.C.L. 219, *Bacillus subtilis* N.R.R.L. 558, *Serratia marcescens* A, *Serratia* *marcescens* B, *Bacillus mycoides* Kräl, *Bacillus mycoides roseus, Pseudomonas fluorescens* N.R.R.L., and *Pseudomonas fluorescens liquefaciens.* The *Escherichia coli* strain is the same one which has been used previously (*4*) for investigation of the nitrate reductase system.

Anaerobic Growth Tests—Anaerobic growth of the aerobic bacteria was obtained according to the following method. After inoculation of the organism into test tubes containing nutrient broth (peptone 1 per cent, meat extract 1 per cent, pH 7.2) with or without nitrate, the tubes were placed in a vacuum desiccator containing solid sodium hydroxide and pyrogallol. The desiccator was evacuated, after which the vessel was filled with oxygen-free nitrogen gas to about 50 mm.-Hg. A small portion of water was carefully introduced to react with the sodium hydroxide and pyrogallol, thus absorbing any remaining oxygen. To confirm the anaerobicity throughout the incubation period, a slant culture of *Bac. subtilis* was placed in the vessel. In none of the experiments could any growth be found in the slant culture of *Bac. subtilis.* Incubation was estimated photoelectrically, any necessary dilution of the medium being made with the same nutrient broth.

Nitrite was estimated colorimetrically as previously described (*5*).

Extent of Nitrate Reduction—The organisms were grown aerobically in nutrient broth (or on broth agar) containing nitrate at 30°. Cells were harvested and washed with water by centrifuging until no color raction of nitrite could be found in the supernatant fluid. They were finally suspended in water and were placed in a Thunberg tube with a known amount of nitrate and a suitable hydrogen donor. Incubation was at 30° under anaerobic conditions. The reaction was stopped by pouring the reaction mixture into an uranyl acetate solution (saturated). After centrifuging, nitrite was estimated as described previously. When leuco methylene blue was used as an electron donor, the dye was removed by kaolin after deproteinization by uranyl acetate. The micro-estimation of nitrate was made according to the enzymatic method (6) using a dried cell preparation of *E. coli* as the enzyme source.

Assay of Radioactivity—Radioactivity of the cells was determined according to the following method. Cells were harvested from the reaction mixture by centrifuging, and were washed four times with water, and finally suspended in water. The suspension thus prepared was placed in a stainless steel dish and was dried with an infrared lamp. Radioactivity of the cells was counted by the conventional Geiger-Müller counter with an end window.

Assay of Inorganic Phosphorus—Inorganic orthophosphate was estimated colorimetrically according to the method of Fiske and Subbarow (*7*).

RESULTS AND DISCUSSION

Growth of Aerobic Bacteria under Anaerobic Conditions in the Presence of Nitrate

Eight strains of aerobic bacteria and *E. coli* were incubated anaerobically at 30° for 20 or 36 hours in the nutrient broth, with or without nitrate. The rates of growth and the accumulation of nitrite in these media are summarized in Table I. As shown, *Bac. subtilis* and *Bac. mycoides* and one strain of *Pseudomonas fluorescens* showed negligible growth with or without nitrate. These bacteria, however, produced appreciable amounts of nitrite when they were grown in nutrient broth containing nitrate under aerobic conditions, which is in accord with the descriptive nature of these organisms (*8*). Since no nitrite could be found in any medium where the bacterium showed no growth, the possibility that nitrite inhibited the growth could be excluded.

TABLE I

Growth of Aerobic Bacteria and E. coli in the Presence of Nitrate under Anaerobic Conditions

Medium used / Organisms	Broth	Broth + 0.5% KNO_3		Broth + 1.0% KNO_3	
	Growth	Growth	Nitrite formed	Growth	Nitrite formed
			μM/ml.		μM/ml.
Serratia marcescens strain A	0.021	0.216	53.0	0.326	71.5
Serratia marcescens strain B	0.045	0.220	51.9	0.326	56.3
Pseudomonas fluorescens liquefaciens	0	0.440	6.1	0.498	29.6
Pseudomonas fluorescens N.R.R.L.*	0	0	0	0	0
Bacillus subtilis P.C.L. 219*	0	0	0	0	0
Bacillus subtilis N.R.R.L. 558	0	0	0	0	0
Bacillus mycoides Kräl*	0	0	0	0	0
*Bacillus mycoides roseus**	0	0	0	0	0
Escherichia coli	0.088	0.260	40.4	0.326	41.9

The rate of growth was expressed by the extinction of the culture fluid estimated at 700 mμ. Data presented are proportional to the dry weight of the cells.

* Estimation was made at 20 hours after inoculation. Other experiments were made at 36 hours.

In accord with the earlier observation (*1*), *E. coli* could grow more rapidly in a medium containing nitrate than in the usual nutrient broth under anaerobic conditions. Two strains of *Serratia* and one strain of

Pseudomonas showed very rapid growth under anaerobic conditions when they were supplied with nitrate. Appreciable amounts of nitrite were accumulated under these conditions. The initial amount of nitrate before sterilization was 49.5 μM/ml. medium (in the case of 0.5 per cent KNO_3), and almost theoretical amounts of nitrite were found in the incubation media of the two strains of *Serratia.* These results suggest that *Serratia marcescens* catalyzes the reduction of nitrate to nitrite but that nitrite reducing activity is not present. On the other hand, in the case of *Pseudomonas,* accumulation of nitrite was rather small, and a considerable amount of gas was formed during incubation. This again agrees with the biochemical nature of this organism, that is, that this bacterium catalyzes the denitrification (*8*).

The above results reveal that nitrate reacts as an obligatory electron acceptor in a facultative anaerobic bacterium (*E. coli*) as well as in some more aerobic bacteria (*Serratia marcescens* and *Pseudomonas fluorescens*). The nitrate reducing activity, however, seems not to be the only factor which is responsible for the bacterial growth in a medium containing nitrate under anaerobic conditions. This may be explained by the multifunctional nature of the nitrate reducing system, in other words, that the role of nitrate reductase in biological systems differs from organism to organism as has been discussed by Taniguchi, Sato, and Egami (*4*).

Some Properties of the Nitrate Reducing Reaction of Aerobic Bacteria

(*A*) *Search for the Suitable Electron Donor for the Reduction of Nitrate in Serratia marcescens Strain B.—Serratia marcescens* B was grown anaerobically in nutrient broth containing 1 per cent KNO_3 at 32° for 36 hours. Cells were harvested by centrifuging, washed, and suspended in water. The formation of nitritie from nitrate in the presence of glucose, formate, and succinate was estimated. Among these electron donors, glucose was found to be the most suitable, as shown in Table II.

In a separate test, leuco methylene blue was found to be a suitable electron donor for the nitrate reduction in this organism. The result is presented in Fig. 1.

(*B*) *Nitrite Reducing Activity of Serratia marcescens B—Serratia marcescens* B was grown on broth agar containing 0.5 per cent KNO_3 for 20 hours at 30°. The reduction of nitrite by the resting cells was estimated in the presence of glucose and of leuco methylene blue as electron

TABLE II

Nitrate Reduction by the Resting Cell of S. marcescens

Additions	Glucose	Formate, KNO_3	Succinate, KNO_3	Glucose, KNO_3
Nitrite formed *μM/lit.*	0	44	0	278

Each vessel contained cells suspension 1.0 ml. (ca. 10 mg. dry weight) and *M*/4 phosphate buffer (pH 7.2) 1.0 ml. in a total volume of 5.0 ml. In each case, 100 μM of hydrogen donor and 10 μM of KNO_3 were added as indicated. Incubation was at 30° for 20 minutes.

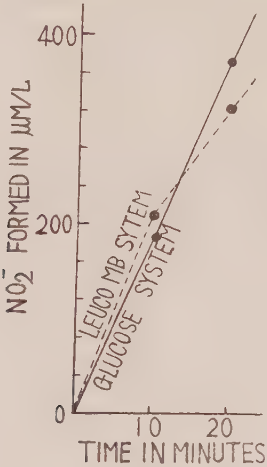

FIG. 1. Nitrate reduction by *S. marcescens.*

Each vessel contained 0.5 ml. (5.2 mg. dry weight) cell suspension, grown aerobically on broth agar plate containing 0.5 per cent KNO_3 at 30° for 20 hours, *M*/4 phosphate buffer (pH 7.2) 1.0 ml., and 10 μM of nitrate in a total volume of 5.0 ml. 100 μM of glucose or 10 μM of leuco methylene blue were used as an electron donor. Incubation was at 30° under anaerobic conditions.

donors. As shown in Table III, the activity of nitrite reduction by these cells was negligible, and furthermore, no oxidation of leuco methylene blue was observed as judged from the coloration of the reaction mixture. Therefore, the conclusion that nitrite is the reduction

TABLE III

Reduction of Nitrite by the Resting cell of S. marcescens

Electron donor	Glucose		Leuco methylene blue	
	At 0 time	After incubation	At 0 time	After incubation
Nitrite found μM/lit.	1778	1630	1814	1852

Each vessel contained cell suspension 0.5 ml. (6.7 mg. dry weight), $M/4$ phosphate buffer (pH 7.2) 1.0 ml. and 10 μM of nitrite in a total volume of 5.0 ml. 100 μM of glucose or 10 μM of leuco methylene blue were added as an electron donor. Incubation was at 30° for 30 minutes under anaerobic conditions.

product of nitrate in this organism is supported not only by the growing culture experiment already discussed, but also by the experiment with resting cells.

(*C*) *Search for a Suitable Hydrogen Donor for the Reduction of Nitrate in Pseudomonas fluorescens liquefaciens*—The organism was grown aerobically on a broth agar plate containing 0.5 per cent KNO_3 at 30° for 20 hours. Resting cells were prepared by the usual method. The formation of nitrite from nitrate was estimated in the presence of glucose, succinate, formate, and leuco methylene blue. In some cases, a small amount of nitrite was estimated, but usually no accumulation of nitrite occurred. Therefore, the reduction of nitrate was estimated by measuring the disappearance of nitrate in the reaction mixture. The results are summarized in Table IV. As shown, formate was found to be the most suitable hydrogen donor, succinate and glucose being inferior.

When the resting cell suspension was kept at —15° and thawed thereafter, considerable disintegration of the cells was observed. The cloudy supernatant solution obtained by centrifugation of a freeze-thawed preparation at 7,000 × *g* for 10 minutes showed nitrate reducing activity in the presence of formate. However, there was no indication of nitrite reduction by this system. A particulate fraction prepared by centrifugation of the above preparation at 15,000 × *g* for 30 minutes displayed nitrate reduction in the presence of formate. The supernatant solution in this step lacked the ability to activate nitrate. Therefore, it is clear that the nitrate reductase, at least in part, is associated with the particulate enzymes of this organism.

(*D*) *Nitrite as An Intermediate in Nitrate Reduction in Pseudomonas*

TABLE IV

Reduction of Nitrate by Pseudomonas fluorescens

Electron donor	None*	None	Glucose	Succinate	Formate	Leuco methylene blue
Nitrate estimated after incubation μM	9.14	4.90	4.03	4.01	2.50	5.26
Nitrate consumed μM	—	4.24	5.06	5.13	6.64	3.88

* Reaction was stopped at zero time.

Each vessel contained 0.5 ml. cell suspension (*ca.* 5.0 mg. dry weight), *M*/4 phosphate buffer (pH 7.2) 1.0 ml. and 10 μM of KNO_3 in a total volume of 5.0 ml. 100 μM of each electron donors were added to the system except leuco methylene blue. 10 μM of leuco methylene blue was added as an electron donor. Incubation was at 30° for 25 minutes under anaerobic conditions.

fluorescens liquefacines—As stated previously, the accumulation of nitrite as a reduction product of nitrate by *Ps. fluorescens* is not observed under the usual experimental conditions. Usually cells displayed higher reducing activity toward nitrite than toward nitrate. However, as stated before, cells which had been frozen at −15° for several hours lost almost all the nitrite reducing activity, and the formation of nitrite from nitrate was easily demonstrated as shown in Fig. 2. Thus, it is clear that nitrite is an intermediate in nitrate reduction in this organism.

(*E*) *The effect of Some Inhibitors on the Nitrate Reducing Activities of Serratia and Pseudomonas*—The effect of several inhibitors on nitrate reduction by resting cells of *Serratia marcescens* and by the preparation from *Pseudomonas fluorescens* is summarized in Table V. In every case in which a metal combining reagent, such as cyanide or azide, was used, it inhibited the reduction of nitrate greatly, thus suggesting the participation of a metal component in each system.

(3) *Incorporation of P^{32}-Labelled Orthophosphate Coupled with the Reduction of Nitrate in Escherichia coli Cells*

In order to elucidate the rate of phosphorus turnover in cells during the reduction of nitrate, a resting cell suspension of *E. coli*, grown anaerobically in nutrient broth with 1.0 per cent KNO_3, was added to P^{32}-labelled orthophosphate, potassium nitrate, and sodium formate·

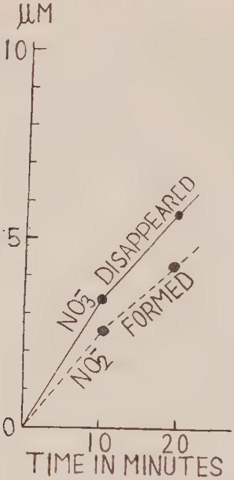

FIG. 2. Formation of nitrite from nitrate by a freeze-thawed preparation from *Pseudomonas fluorescens.*

Each vessel contained 0.5 ml. suspension (*ca.* 5 mg. dry weight), *M*/4 phosphate buffer (pH 7.2) 1.0 ml., 100 μM of formate, and 10 μM of nitrate in a total volume of 5.0 ml. Incubation was at 30° under anaerobic conditions.

TABLE V

Effect of Several Inhibitors on Nitrate Reduction by S. marcescens and Ps. fluorescens

Agents	Concentration of inhibitor (*M*)	Inhibition (%)	
		*S. marcescens**	*Ps. fluorescens***
KCN	10^{-3}	100	100
NaN_3	10^{-3}	100	100
Versene	1.4×10^{-3}	0	10.4
α,α′-Dipyridyl	10^{-3}	13.8	21.3
CO	1 atom	0	10.4

* Leuco methylene blue was used as a hydrogen donor.

** Freeze-thawed preparation was used. Hydrogen donor was formate.

The reaction was carried out at 30° for 40 minutes under anaerobic conditions. After incubation, the cells were removed by centrifugation and the radioactivity of the cells was estimated. Inorganic phosphate and nitrite in the reaction medium were estimated as previously described. Phosphorus turnover accompanied by the oxidation of formate by molecular oxygen was also examined to compare the rate of exchange with that occurring when nitrate was supplied as electron acceptor. The composition of the media and the analytical data are presented in Tables VI and VII, respectively.

TABLE VI

Composition of the Reaction Media

Experimental number	1	2	3	4	5	6
Conditions of reaction	Aerobic		Anaerobic			
Cell suspension* *ml.*	2.0	2.0	2.0	2.0	2.0	2.0
P^{32} solution** *ml.*	0.4	0.4	0.4	0.4	0.4	0.4
M/5 Sodium formate *ml.*	1.4	0	1.4	1.4	0	0
1 *M* Potassium nitrate *ml.*	0	0	0.2	0	0.2	0
H_2O *ml.*	0.2	1.6	0	0.2	1.4	1.6

* 26.2 mg. dry weight.

** The solution contained 25 μM of orthophosphate and the total radioactivity was 1.04×10^5 counts per minute.

TABLE VII

Incorporation of P^{32}-Orthophosphate Coupled with the Reduction of Nitrate

Experimental number	1	2	3	4	5	6
Oxygen uptake μM	58.3	0	—	—	—	—
Nitrite produced μM	—	—	167.2	0.7	2.1	0.3
Inorganic phosphate in the medium after incubation μM	24.5	24.8	25.2	24.8	25.3	25.4
Specific activity of the cells *cpm/mg. cells*	189.5	109.5	143.8	107.0	109.8	94.2
Increase in P turnover %	73.1	—	52.7	11.4	16.6	—

As shown in Table VII, the oxidation of formate by molecular oxygen caused an increase in phosphorus turnover. This, of course, is attributed to oxidative phosphorylation. Appreciable incorporation of P^{32} was observed when nitrate was supplied as an ultimate electron acceptor. Omission of either nitrate or formate from the system decreased the incorporation significantly. Although these results do not provide unequivocal demonstration of the occurrence of the phosphorylation reaction at the site of the electron transport system associated with the reduction of nitrate to nitrite, it is highly probable that such a coupled system is present in *E. coli* cells. Worthy of note is the recent finding of Taniguchi, Sato and Egami (*4*) that nitrate reduction in an *E. coli* extract is inhibited by 2-heptyl-4-hydroxyquinoline-*N*-oxide which had been shown by Lightbown and Jackson to inhibit the oxidation of cytochromes b and b_1 in animal tissues and bacteria (*9*).

SUMMARY

Among the eight strains of aerobic bacteria tested, two strains of *Serratia marcescens* and one strain of *Pseudomonas fluorescens* were found to show rapid growth in a nitrate-containing nutrient broth medium under strictly anaerobic conditions. The other five strains, which are *Bacillus subtilis* (2 strains), *Bacillus mycoides* (2 strains), and one strain of *Pseudomonas fluorescens* showed no growth in the same medium under the same conditions. The latter group of bacteria, however, grew well and produced appreciable amount of nitrite in a nitrate medium under aerobic conditions, suggesting the existence of a nitrate activating enzyme system. These results might be explained by the multifunctional nature of the nitrate reductase system in microorganisms.

Some properties of the nitrate reducing systems in *Serratia* and *Pseudomonas*, which showed typical nitrate respiration, were investigated. Resting cells of *Serratia* reduced nitrate to nitrite in the presence of either glucose or leuco methylene blue as an electron donor. On the other hand, *Pseudomonas* cells reduced nitrate as well as nitrite in the presence of formate but not of leuco methylene blue as a hydrogen donor. In both the *Serratia* and *Pseudomonas* systems, the nitrate reducing activity is sensitive to metal chelating agents, suggesting a metalic component in these systems.

Phosphorus turnover in resting cells of *E. coli*, which also showed nitrate respiration, was greatly enhanced by the addition of nitrate and formate under anaerobic conditions. When either formate or nitrate was omitted from the system, the rate of turnover significantly decreased. The results, though indirect, may suggest the occurrence

of coupled phosphorylation to the electron transport system between formic dehydrogenese and nitrate reductase.

REFERENCES

(*1*) Quastel, J. H., Stephenson, M., and Whetham, M. D., *Biochem. J.*, **19**, 304 (1925)

(*2*) Aubel, E., and Egami, F., *C. R. Acad. Sci.*, **202**, 675 (1936)

(*3*) Sato, R., *Scienca Revuo.*, **2**, 122 (1950)

(*4*) Taniguchi, S., Sato, R., and Egami, F., Inorganic nitrogen metabolism, edited by McElroy, W. D., and Glass, B., The Johns Hopkins Press pp. 87–108 (1956)

(*5*) Taniguchi, S., Mitsui, H., Toyoda, J., Yamada, T., and Egami, F., *J. Biochem.* (*Japan*), **40**, 175 (1953)

(*6*) Egami, F., Iida, K., Dôke, T., and Taniguchi, S., *Bull. Chem. Soc. Japan*, **27**, 619 (1954)

(*7*) Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925)

(*8*) Bergey, D. H., Breed, R. S., Murray, E.G.D., and Hitchens, A. P. Bergey's Manual of determinative bacteriology, 5th Ed. Williams and Wilkins, Baltimore (1939)

(*9*) Lightbown, J. W., and Jackson, F. L., *Biochem. J.*, **58**, xlix (1954)

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INTERRELATION BETWEEN THE FUNCTION OF HEME-PROTEINS AND THE STRUCTURAL MODIFICATIONS OF THEIR PROTEIN PARTS

VI. STUDIES ON THE REACTIONS OF MODIFIED METHEMOGLOBINS WITH CYANIDE

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(Received for publication, January 24, 1956)

As reported by the present author (*1*), when benzoate or salicylate was added in certain concentrations into hemoglobin solution, the protein moiety of the latter was affected to be reversibly denatured or structurally modified, so that another globin-N became accessible to bonding to heme-Fe forming hemichrome or hemochrome structure which could be evidenced spectroscopically. Further, from this laboratory, Kikuchi *et al.* (*2*) reported that with much lower concentrations of benzoate or salicylate, the oxidative activity of hemoglobin was strongly promoted, though any of its structural modifications could be proved spectroscopically. In higher concentration range of these perturbators, the modification proceeded more profoundly to spectroscopically visible degree in forming hemichrome structure as mentioned above, the oxidative activity vanished completely. These results and those of electrophoretic investigations made also by the present author (*3*) suggest that the protein modification in the hemoglobin molecule induced by these perturbators as benzoate, salicylate, laurate, dodecylsulfate *etc.* is a stepwise process.

From another site, Jung (*4*) reported a decrease of the combining affinity of cyanide toward methemoglobin when the latter was reversibly denatured at the pH of 4.3.

From his interest on the interrelation between the structural and functional modifications of hemoglobin, the present author intended to study the influence of the structural modifications of protein part upon the combining affinity of globin-N groups in modified methemoglobin toward heme Fe^{III}. The present paper deals with the competitive binding of globin-N groups with cyanide molecules toward heme Fe.

EXPERIMENTAL

Crystalline equine oxyhemoglobin for the experimental purpose was prepared following the method of Suzuki *et al.* and was dissolved in distilled water. Sodium nitrite, twice as much as heme concentration, was then added to this solution in order to convert hemoglobin oxidatively to methemoglobin. After the completion of the oxidation, remaining nitrite was removed by dialysis against distilled water. The concentration of methemoglobin solution was determined by converting into alkali denatured globin hemochrome, the value of $E_{mM}=30.6$ at 558 mμ for the latter being applied. 2.5 M solution of benzoate and 2×10^{-3} M solution of sodium dodecyl sulfate were prepared as the stock solution, from which the solutions of desired concentrations were prepared by dilution every time at use. pH of these solutions were adjusted to 8.0 by use of a glass electrode.

To equalize the ionic strength of a solution of sodium dodecyl sulfate with benzoate solution, KCl solution was used. The KCN solutions of desired concentrations were prepared by dilution of 0.1 M KCN stock solution with 0.1 M KCl solution.

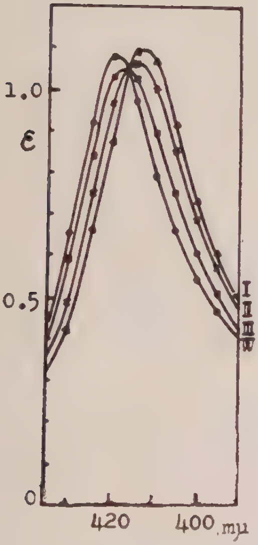
Procedure: Into small beakers were placed 2.5 ml. of methemoglobin solution, 1.5 ml. of 0.1 M phosphate buffer (pH 8.0) and 5.0 ml. of sodium benzoate or sodium dodecyl sulfate solution, then were kept about 10 minutes at room temperature. After incubation, the KCN solutions in varied concentrations were added to the above mixture. The absorption spectra of these reaction solutions were measured by a photoelectric spectrophotometer Model EPB-V of Hitachi and Co. In these experiments, the absorption measurements were restricted within the Soret region, for the absorption spectrum of CN-methemoglobin in the visible region is almost indistinguishable from that of dicyanhematin.

RESULTS AND DISCUSSION

1. *Reaction of Hemichrome (Induced by Benzoate) with Cyanide*—After the addition of benzoate to methemoglobin solution in the final concentration of the former at 1.25 M, an absorption figure of hemichrome was obtained as given in Fig. 1, I. Into this hemichrome solution KCN solution was added in varied concentrations as described in Fig. 1; a series of different absorption figures were then obtained. A limit of absorption change was attained at the KCN concentration of 5×10^{-2} M as shown in Fig. 1, IV. Even after the addition of KCN in the concentration at 2.0 M, no further absorption change were observed. Thus this figure may be regarded as the final limit of combining product of hemichrome with cyanide. Further, an isosbestic point was clearly demonstrated in the figure, indicating a reaction system of two components consisting of hemichrome and cyanide compounds.

Based on the absorption changes, the percentage of cyanide compound formation at each KCN concentration was calculated from the nomogram of the wavelength at 425 mμ, by use of which the percentages of cyanide compound formation were obtainable directly from extinction at any wavelength. These values were plotted against the log concentrations of cyanide as shown in Fig. 2. The curve in Fig. 2 is the theoretical sigmoid curve of first order. The experimental data fit well to this theoretical curve. Based on these experimental data, the reaction of hemichrome with cyanide can be expressed by the following equation.

$$\mathrm{Fe^{III}}{<}{\substack{\mathrm{gl.}\\ \mathrm{gl.}}} + \mathrm{CN^-} \rightleftharpoons \mathrm{Fe^{III}}{<}{\substack{\mathrm{gl.}\\ \mathrm{CN}}} + \mathrm{gl.}$$



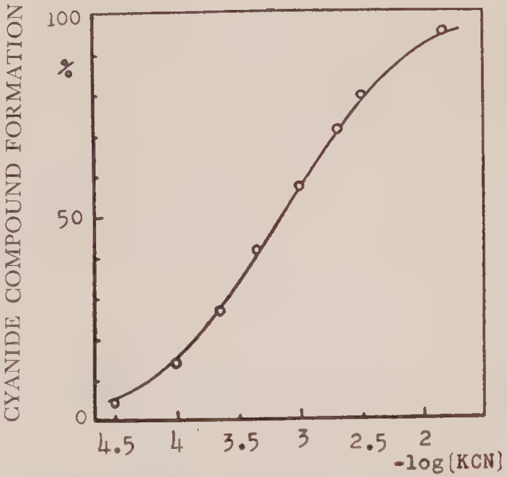


FIG. 1. Absorption changes of hemichrome (induced by benzoate) by addition of cyanide in varied concentrations.

Concentration of methemoglobin: 8.63×10^{-6} *M*
Concentration of benzoate : 1.25 *M*
Concentrations of KCN : I. 0 *M* II. 2×10^{-4} *M*
III. 1.5×10^{-3} *M* IV. 5×10^{-2} *M*
pH=8.0 (phosphate buffer) T=16°

FIG. 2. Plots of the percentages of cyanide compound formation against log concentrations of cyanide.

$Fe^{III}<^{gl.}_{gl.}$ shows a modfied globin hemichrome structure. One of the two globin-N groups became accessible to combine with heme Fe^{III} through the unfolding of the peptide chain in the modified globin moiety. Since this reaction proceeds as a first order reaction, it may be assumed that either of these two globin-N groups can be substituted for cyanide. The absorption figure of this cyanide compound was similar to that of cyanmethemoglobin. It may thus be assumed that this cyanide compound and the cyanmethemoglobin may both have a same structure in respect to a binding of cyanide and globin-N group for heme Fe^{III}.

II. Reactions of Hemichrome (Induced by SDS) with Cyanide—By addition of sodium dodecyl sulfate (SDS) to methemoglobin solution in the final concentration of the former at 10^{-3} *M*, an absorption figure of hemichrome similar to that obtained by addition of benzoate was observed as shown in Fig. 3, I. Thus it may be assumed that the hemichrome structure induced by SDS is similar to that of hemichrome induced by benzoate.

This absorption figure of hemichrome (Fig. 3, I) changes by addition of KCN in varied concentrations so that a series of different absorption figures were obtained as shown in Fig. 3. Fig. 3, IV is the limit of the absorption change. No further change of absorption figure were observable even by excessive KCN. The absorption figure 3, IV can thus be regarded as the final limit of reaction product of hemichrome with cyanide.

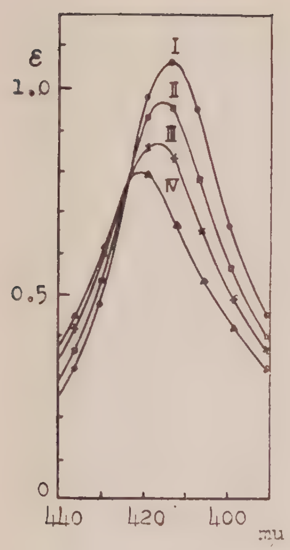
It is notable that Fig. 1, IV and Fig. 3, IV are not the same. But as an isosbestic point was clearly demonstrated in a series of absorption changes, the reaction system is surely of two components consisting of hemichrome and cyanide compound.

From this absorption changes, the percentages of cyanide compound formation were calculated by a nomogram. The plot of percentages of cyanide compound against the log concentrations of cyanide fitting to the theoretical curve of second order is shown in Fig. 4.

From this, the reaction of SDS denatured globin hemichrome with cyanide can be expressed by the following equation.

$$Fe^{III}<^{gl.}_{gl.} + 2\,CN^- \rightleftharpoons Fe^{III}<^{CN}_{CN} + 2\,gl.$$

Then the two globin-N groups linking to heme Fe^{III} were substituted in this case simultaneously for two cyanide molecules. Thus the reaction product must be dicyanhematin. In another experiments,



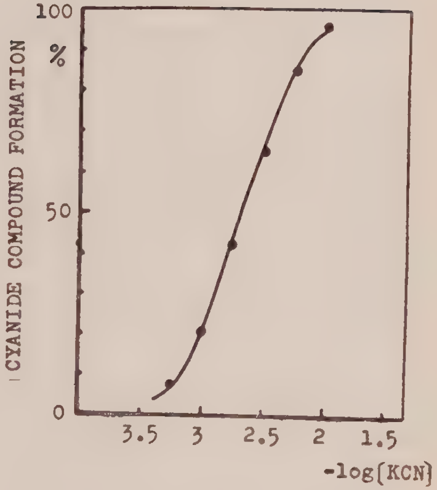


FIG. 3. Absorption changes of hemichrome (induced by SDS) by addition of cyanide in varied concentrations.

Concentration of methemoglobin: 8.63×10^{-6} *M*
Concentration of SDS : 10^{-3} *M*
Concentrations of KCN : I. 0 *M* II. 1.8×10^{-3} *M* III. 3×10^{-3} *M* IV. 5×10^{-2} *M*
pH=8.0 T=16°

FIG. 4. Plots of the percentages of cyanide compound formation against log concentrations of cyanide.

it was proved that the absorption figure of dicyanhematin obtainable by addition of excess cyanide to alkali hematin was similar to that of dicyanide compound of SDS denatured globin hemichrome.

In spite of the similarity of absorption figures of these two hemichromes, they behave in different manner in the reaction process of hemichrome with cyanide. Namely, the two globin-N groups of SDS denatured globin hemichrome are substituted simultaneously for two cyanide molecules, while in the benzoate denatured globin hemichrome solely one of the two globin-N groups is substituted for cyanide.

It is impossible from the experimental data to decide which of these two globin-N groups in the benzoate denatured globin hemichrome may be substituted for cyanide. But from the fact that, in native methemoglobin the globin-N group linking to heme Fe^{III} is not substitutable for cyanide, and on the other hand, on the consideration that the modification by benzoate may be of a slight grade, it may be more plausible that the secondary linking globin-N is substituted for cyanide.

Horiguchi (*5*) reported that in an alkali denatured globin hemichrome, its two globin-N groups were substituted for cyanides. As demonstrated in the present experiment, also in SDS denatured globin hemichrome, its two globin-N groups are substituted for cyanide. This similarity in respect to the substitution reaction suggests that the essential globin-N group in native methemoglobin may become substitutable, as well as anoter globin-N group, when the protein modification proceeded more profoundly.

It is of interset that the substitution reaction in alkali denatured globin hemochrome proceeds in two steps in contrast to the one step reaction in SDS denatured globin hemichrome. For the elucidation of this discrepancy, further experiments are necessary.

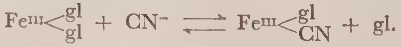
Mizutani (*6*) in this laboratory indicated that the combining affinity of globin-N group for heme Fe decreased after its protein part was partially digested by pepsin.

From all of these experimental facts, it was demonstrated that the combining affinity of globin-N groups to heme-Fe is affected not only by a more drastic modification of chemical structure as by pepsin digestion as well as by irreversible denaturation in the conventional meaning, but also by several modifications of milder grade. In the latter case, the affinity alters in different ways and grades according to the difference of perturbators and to the nature and the grade of the structural modifications. For the more precise elucidation of the reaction taking place in these protein modifications and its effect to the affinity of globin-N groups toward heme-Fe, much remains to be investigated.

SUMMARY

Two hemichromes, obtained by addition of benzoate and sodium dodecyl sulfate, react with cyanide according to the following equations, respectively.

1)



$$Fe^{III}\Big\langle\begin{smallmatrix}gl\\gl\end{smallmatrix} + CN^- \rightleftharpoons Fe^{III}\Big\langle\begin{smallmatrix}gl\\CN\end{smallmatrix} + gl.$$

2)

$$\mathrm{Fe^{III}}{<}^{\mathrm{gl}}_{\mathrm{gl}} + 2\,\mathrm{CN^-} \rightleftharpoons \mathrm{Fe^{III}}{<}^{\mathrm{CN}}_{\mathrm{CN}} + 2\,\mathrm{gl}.$$

Based on these results, some discussions were made on the interrelation between the structural modifications of globin part and the affinity of globin-N group toward heme Fe.

The present investigation was supported by Prof. K. Kaziro, to whom the author's deep gratitude is due. I should like to express my thanks to Dr. G. Kikuchi for his helpful discussion during the course of this work. The author thanks also to the Ministry of Education for the Grant in Aid for Scientific Research. (K. Kaziro).

REFERENCES

(*1*) Tsushima, K., *J. Biochem.*, **41**, 215 (1954)
(*2*) Kikuchi, G., and T. Tomimura, *J. Biochem.*, **41**, 503 (1954)
(*3*) Tsushima, K., and M. Kawai, *J. Biochem.*, **43**, 13 (1956)
(*4*) Scheler, W., F. Jung, and K. H. Hoffmann, *Biochem. Z.*, **325**, 401 (1954)
(*5*) Horiguchi, M., *J. Jap. Biochem, Soc.*, **25**, 225 (1954)
(*6*) Mizutani, H., *J. Jap. Biochem. Soc.*, **27**, 550 (1956)

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INTERRELATION BETWEEN THE FUNCTION OF HEME-PROTEINS AND THE STRUCTURAL MODIFICATIONS OF THEIR PROTEIN PARTS

VII. SULFHYDRYL GROUP OF MODIFIED METHEMOGLOBIN

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As reported by Tsushima (*1*) in our laboratory, when benzoate or salicylate in certain concentration was added to hemoglobin solution, absorption figure of hemoglobin was changed to that of hemochrome or hemichrome. This was assumed by Tsushima to be the result of a structural modification of globin part in the hemoglobin molecule (perturbation in the meaning of Holden (*2*)). By the effect of these agents (perturbators), some part of its globin-N which has so far been buried in the inner molecule of hemoglobin seems to be exposed or unmasked, so that one of these globin-N may thus become accessible to the bonding of heme-Fe to its sixth coordination. This compound may be then termed chemically as hemochrome (Fe^{II}) or hemichrome (Fe^{III}), showing optically a hemochrome or hemichrome figure as the fact. This sort of structural change of globin moiety has been proved by Tsushima to be of reversible one. On the other hand, the essential function of hemoglobin to combine with oxygen is lost by the addition of perturbators, and instead, as reported by Kikuchi *et al.* (*3*), it shows then a pronounced activity as an oxidase, in so far as the concentration of perturbators was kept relatively in lower range. In more progressed state of perturbation caused by a higher level of perturbators, a hemichrome figure being formed as above stated, this oxidase activity is lost. Also the function of catalase can be modified by perturbators as reported by Suzuki and Tomimura (*4*). The results of these investigations suggest the possible structural modification of protein moiety of these heme-proteins through the fact that all of these heme-proteins were functionally modified by the perturbators. But the substantial basis of these structural modification is not yet elucidated.

The present author intended to study the nature of these protein modifications by benzoate by means of the determination of the SH groups in the hemoglobin molecule whether they can be exposed from its buried state through these structural modifications.

EXPERIMENTAL

Pure ferricyanide used in the present experiment was prepared ferrocyanide free by recrystallization of the Merck preparation.

Oxyhemoglobin solution was prepared from crystalline equine oxyhemoglobin (free from methemoglobin) prepared by the method of Suzuki *et al.* (*5*), which was dissolved in distilled water. The concentration of oxyhemoglobin solution was determined by the alkali denatured globin hemochrome method.

Optical densities were estimated by the use of photoelectric spectrophotometer Model EPB-V of Hitachi and Co. Cuvette of 10 mm. path length was used in the measurement.

Determination of SH groups in the native and modified hemoglobin molecule was performed as follows. SH groups in the hemoglobin molecule was determined by the reduction of ferricyanide. For the estimation of ferricyanide, as previously described by the author (*6*), optical density at 420 mμ was measured and $\varepsilon_{\mathrm{mM}}^{420}=1.08$ (as shown by the calibration curve, Fig. 1) was applied to obtain the absolute concentration.

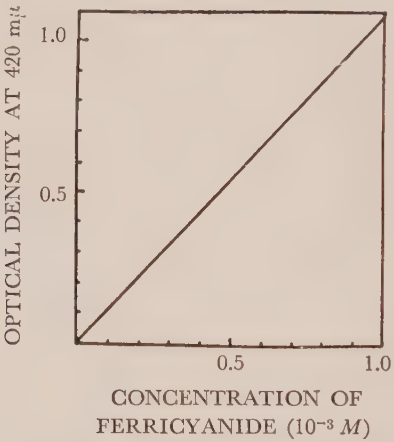

Fig. 1. Calibration curve of ferricyanide. Optical density of ferricyanide is not altered by pH change. $\varepsilon_{\mathrm{mM}}^{420}$ of ferrocyanide, estimated as 0.01, is practically negligeble.

I. SH Groups in the Native Oxyhemoglobin Molecule—When ferricyanide was added to oxyhemoglobin solution, both of its heme Fe^{II} and freely reacting SH groups are oxidized, hemoglobin being converted into methemoglobin. In the reaction of oxyhemoglobin with ferricyanide about 10 times as much as heme concentration, the oxidation in forming methemoglobin completed within 10 minutes at pH 7.38 and room temperature (Fig. 2). The figure was obtained by measuring the decrease in the optical density at 578 mμ. When ferricyanide twice as much as heme concentration was used, 60 minutes were needed for the completion of the oxidation. When the oxidation was in completion, the optical density at 578 mμ being stabilized, the methemoglobin produced was estimated in the form of CN-methemoglobin. The amount of methemoglobin found coincided well with the initial amount of oxyhemoglobin.

Oxygen-output during the oxidation was also measured by the use of Warburg manometer at room temperature. As may be seen

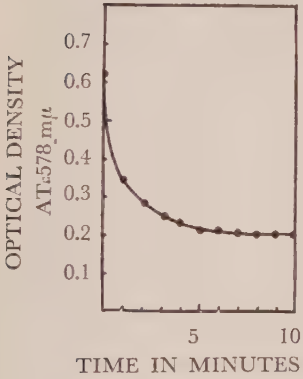

FIG. 2. Velocity of oxyhemoglobin oxidation.

Oxyhemoglobin 1.2×10^{-4} M (heme concentration)	1 ml.
Phosphate buffer $M/15$ (pH 7.38)	1 ml.
Ferricyanide 1.2×10^{-3} M	1 ml.

Optical density at zero time was determined by control mixture, including 1 ml. of water instead of ferricyanide.

TABLE I

Oxygen-output from Oxyhemoglobin by Ferricyanide Oxidation

pH	6.82	7.38*	7.85	9.35*	Mean
O_2-output (a) $\mu l.$	36.0	21.8	34.5	22.1	—
Theoretical value (A) $\mu l.$	35.7	23.5	35.7	23.5	—
(a)/(A)	1.07	0.93	0.97	0.94	0.97

Oxyhemoglobin 5.3×10^{-4} M (heme concentration) 3 ml.
Buffer ($M/15$ phosphate buffer or $M/5$ carbonate buffer) 2 ml.
Ferricyanide 1.0×10^{-2} M 0.5 ml.
* Oxyhemoglobin 3.5×10^{-4} M (heme concentration) was used in this case.

in Table I, all the oxygen-outputs at varied pH were in good agreement with the theoretical value calculated from the optically determined amount of oxyhemoglobin. The effect of pH upon the amount of oxygen-output was found thus to be negligible, though the reactivity of SH group is significantly elevated in the alkaline side. This indicates that the SH group in the hemoglobin molecule is fairly stable against oxygen generated from oxyhemoglobin by oxidation even in the alkaline side.

In order to determine the number of freely reacting SH groups in the native oxyhemoglobin molecule, ferricyanide was added several times as much as the amount of heme at room temperature. After the reaction of one hour, the solution was deproteinized with 2 *N* trichloroacetic acid, filtrated by iron free paper treated with diluted HCl, and optical density at 420 mμ of the filtrate was measured. Based on the optical density measured, total consumption of ferricyanide was determined. From their value, the value calculated from the oxidation of heme Fe^{II} was subtracted. Deviding this value by moles of oxyhemoglobin, the number of freely reacting SH groups per mole of oxyhemoglobin can be calculatively obtained.

The determination was carried out with pure crystalline oxyhemoglobin of horse free from methemoglobin. The result is shown in Fig. 3. The number of SH groups was thus found to be 0.75 (approximately one) in average at pH 6–8. As seen in Fig. 3, the number of freely reacting SH groups was found to be much greater when the oxidation was performed at the pH above 8.5. This seems to be due to the more profound unfolding of the protein moiety through its alkali denaturation, not only its SH groups but also its tyrosine phenols being exposed.

The number of freely reacting SH groups in native globin obtained from oxyhemoglobin of horse has been reported by Anson and Mirsky (7) to be 2, determined by the nitroprusside method. The mentioned value, however, was obtained by them only within more alkaline side, namely at pH 9.5. They have found the SH number to be one in weakly alkaline side at pH 7–8, none at pH 6.8 and two at pH 9.5. The SH number of two adopted by them was based on the experimental fact that the SH number of two reversed to zero when the globin solution kept at pH 9.5 was acidified to neutral range. It seems to be possible that hemoglobin molecule in its solution may reversibly be in partially unfolded state at pH 9.5, so that some part of the masked SH groups may have been exposed. The hemoglobin molecule, at least in its solution at pH 9.5 may surely be not in native state.

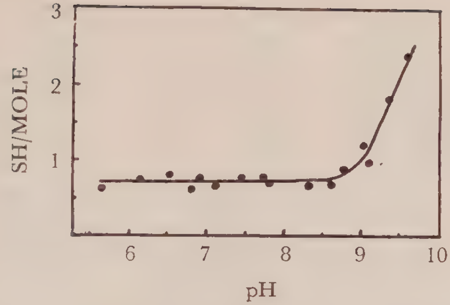

FIG. 3. Freely reacting SH groups in oxhemoglobin.

Oxyhemoglobin 3.7×10^{-3} *M* (heme concentration)	5 ml.
Buffer (*M*/15 phosphate buffer or *M*/5 carbonate buffer)	5 ml.
Ferricyanide 4.0×10^{-3} *M*	10 ml.

After the reaction of 1 hour at room temperature, the reaction mixture was deproteinized with equal volume of 2 *N* trichloroacetic acid. Total ferricyanide consumption was estimated from the decrease of optical density at 420 mμ of the filtrate. SH number/mole was calculated from the following euqation:

SH number=(total ferricyanide consumption − ferricyanide consumed for oxidation of heme) / moles HbO_2 (1/4 heme concentration).

II. The number of SH Groups in the Methemoglobin Molecule Modified by Sodium Benzoate—As described in Fig. 4, ferricyanide solution was added into oxyhemoglobin solution and after the completion of the conversion into methemoglobin, benzoate solutions in varied concentrations were added. After the completion of perturbation confirmed by the spectral changes, the methemoglobin was removed by deproteinization with 2 *N* trichloroacetic acid solution and the remaining ferricyanide in the filtrate was determined photometrically. It was confirmed preliminary that benzoate does not reduce ferricyanide. Though the milli molar extinction coefficient of benzoate at 420 mμ was found to be 0.05, most of the latter is precipitated by the deproteinizing procedure with trichloroacetic acid so that the estimation is not disturbed. Ferricyanide is not to be absorbed by the precipitates of protein nor of benzoate; it can be completely recovered in the filtrate.

The number of the SH groups unmasked were calculated, as above described, from the reduction of ferricyanide. Now, increased SH groups thus exposed per mole of methemoglobin through its modification by benzoate were plotted against the concentration of benzoate added

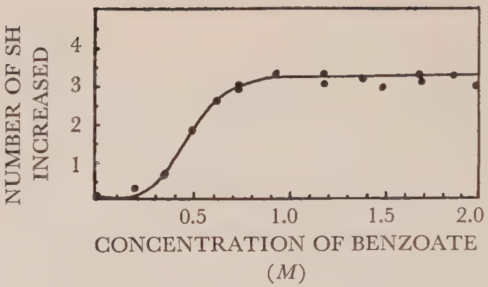

Fig. 4. Increase in SH groups of methemoglobin after its modification by benzoate.

Ten ml. of 9.0×10^{-3} *M* ferricyanide was incubated with 5 ml. of 3.6×10^{-3} *M* oxyhemoglobin (heme concentration) for 1 hour at room temperature. To this solution, 30 ml. of Na benzoate in varied concentrations was added. After standing for 1 hour to complete the hemichrome formation, the reaction mixture was deproteinized with equal volume of 2 *N* trichloroacetic acid. Original SH value has been subtracted from the total SH value.

(Fig. 4). As seen from the figure, the maximum number of increased SH groups was found to be three in average. This was attained at the benzoate concentration of 0.9 *M*. Above this benzoate concentration, no more increase of SH group was found. Taking this maximum value of three as 100 per cent increase, the percentages of increase of SH groups were plotted against the log concentration of benzoate as shown by full circles in Fig. 5. These plots coincided fairly well with the curve, given in full line in the same figure, indicating the relation between hemichrome formation in per cent and the log concentration of benzoate, which was spectrophotometrically obtained by Tsushima (*1*).

It was revealed from this result, that the reversible structural modification of the protein moiety of methemoglobin yielding the formation of hemichrome structure is accompanied by the increase in the SH groups (three in this case) through the unfolding of the molecule. It may thus be concluded that the protein structure in the molecule of methemoglobin yielding hemichrome structure is that with its three SH groups exposed by peptide unfolding, as shown by the plots of two experiments coinciding fairly well with each other.

By these observations it is proved directly that unfolding of the globin part of methemoglobin is actually taking place through the effect of benzoate addition.

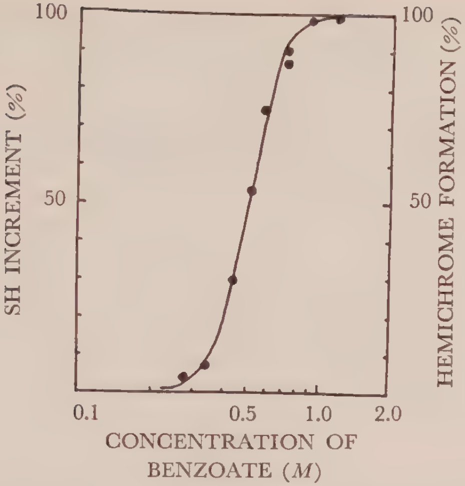

FIG. 5. Relation of SH group increment to hemichrome formation, caused by benzoate.

Full line: curve of hemichrome formation (Tsushima).

● : SH group increment calculated from Fig. 4.

SUMMARY

1. Native oxyhemoglobin of horse posesses one SH group freely reacting by the ferricyanide method.

2. An increase in the number of SH groups in the molecule of methemoglobin could be proved by the addition of benzoate in certain concentrations.

3. Maximum increase of SH groups were found to be three in number at the concentration of benzoate above 0.9 *M*.

4. The concentration range of benzoate yielding either the increase of SH groups and the formation of hemichrome structure coincided fairly well with each other.

The author wishes to express his thanks to Prof. K. Kaziro, Dr. G. Kikuchi, Dr. R. Shukuya and Dr. K. Tsushima for the valuable advice, criticism and encouragement which they have given during the course of this work. Thanks are also due to the Scientific Research Fund of the Ministry of Education for grant.

REFERENCES

(*1*) Tsuhima, K., *J. Biochem.*, **41**, 215 (1954)

(2) Holden, H. F., *Australian J. Exptl. Biol. Med. Sci.*, **25**, 47 (1947)
(3) Kikuchi, G. and Tomimura, T., *J. Biochem.*, **41**, 503 (1954)
(4) Suzuki, M., Tomimura, T., and Mizutani, H., *J. Biochem.*, **42**, 99 (1955)
(5) Suzuki, M., Kajita, A., and Hanaoka, C., *J. Biochem.*, **41**, 401 (1954)
(6) Kajita, A., *J. Jap. Biochem. Soc.*, **26**, 547 (1954)
(7) Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, **19**, 439 (1936)

The Journal of Biochemistry, Vol. 43, No. 2, 1956

FORMATION OF AMYLASE IN A CELL-FREE ENZYME PREPARATION

Dear Sirs:

Despite much effort by many workers, the mechanism of the biosynthesis of protein is as yet unknown, owing to the lack of a suitable experimental system which can synthesize protein in a cell-free state. Recently Gale and Folkes have succeeded in obtaining such a system by the disruption of *Staphylococcus* cells by exposure to supersonic vibration (*1*). Much progress has since been made in this field, but the major problems remain still unsolved.

While we were studying the mechanism of amylase formation by *Bacillus subtilis* H it was found that amylase formation and anaerobic lysis take place in the same growth phase, usually after cell multipication is finished (*2*). In the course of subsequent studies on this lytic phenomenon it was found that the protoplast of this bacterium can be produced by treatment with lysozyme in the presence of a high concentration of sucrose or polyethylene glycol (PEG). We tried to obtain protoplasts capable of synthesizing amylase from cells in the phase of active amylase production, but such protoplasts were unstable and these preparations contained a large amount of lysed cell material. It was found that under certain conditions amylase is more easily formed in the completely lysed cell-free preparation than in "protoplasts" or in intact cells.

Cells were grown in the medium suitable for amylase production: (A) Soy medium (*2*), which contains a high concentration of starch (8 per cent) and an alkaline extract of soybean, in the experiment 1, or (B) GBY medium (glucose 0.5 per cent, peptone 1 per cent, meat extract 0.5 per cent, yeast extract 0.2 per cent, NaCl 0.2 per cent, pH 7.2) fortified with a high concentration of phsosphate (0.15 M) and PEG (10 per cent (*v.*/*v.*), average mol. wt.=400) in experiment 2 (high concentration of PEG was found previously to increase amylase production considerably (3)). After washing with $M/30$ phosphate buffer (pH 7.2) which contained PEG (15 per cent), cells were suspended in the same buffer, treated with lysozyme (28 γ/ml.) at 30° for 25 min. in Expt. 1 or 35 minutes in Expt. 2. Control cell suspensions were not treated. One ml. of these suspensions was added to 10 ml. each of the various media and the formation of amylase was followed. Amylase was determined by the method of Hagihara (*4*) (1 mg. N of crystalline amylase of this bacterium had an activity of 37,000 units). It has been found previously that the boiled extract (100°, 15 to 20 minutes) of cells at the stage of active amylase production greatly promoted the formation of amylase in intact cells (*5*). Therefore boiled cell suspensions prepared in this way were added to the medium. The results of typical experiments are shown in Table I.

TABLE I

Formation of Amylase by Bacillus subtilis H Treated with Lysozyme

Flask no.			1	2	3	4	5	6	7	8
Cells			Control intact-cell suspension				Treated with lysozyme			
							"protoplasts"		Cell-free preparation	
PEG			+		−		+		−	
Boiled cell suspension			−	+	−	+	−	+	−	+
Exptl. no.	Incubation		Amount of amylase formed in units*							
	Aeration	Time								
1	Aerobic	2 hrs.	3.5	9.0	3.9	5.1	3.1	5.8	3.6	6.3
2	Aerobic	4 hrs.	2.2	10.3			1.2	5.8	0	8.4
		8 hrs.	2.7	16.3			2.1	15.0	0	12.8
	Anaerobic	4 hrs.	0	0			0	0	0	5.6

One ml. of the cell suspensions, treated or not treated with lysozyme, was added to 10 ml. of each of the various media which contained, in addition to phosphate ($M/30$, pH 7.2), boiled cell suspension and PEG (15%) as indicated in the table. Figures express the amount of amylase formed in units. 0 means less than 1.0 unit. The reaction was allowed to proceed at 30° under aeration by gentle shaking (aerobic) or without shaking (anaerobic).

* Initial amylase activity was less than 1 unit.

The formation of amylase was clearly demonstrated in the completely lysed cell-free preparation when boiled cell suspension was added. It can be seen from Table I that under fairly anaerobic conditions the formation of amylase was pronounced only in the cell-free preparation while no appreciable amount of amylase was formed in the control cells or in cells in the " protoplast " state. Also under aerobic conditions the amylase formation in response to the boiled cell suspension was sometimes greater in the lysed state than in the untreated state in the early phase of incubation (*e.g.*, see Expt. 1). However, when incubation was continued, amylase production by the untreated cells increased considerably. From the results described above, it is clear that the formation of amylase in this lysed cell preparation is not due to the remaining living cells. In preliminary experiments the active principle in the boiled cell extract was fractionated and shown to be precipitated with three volumes of ethanol. The addition of glucose (1 per cent) and an amino acid mixutre (trypsin digest of casein, 4 mg./ml.) had no effect and the nature of this system seems to be quite different from that of the *Staphylococcus* system.

Further studies on the formation of amylase in this cell-free system are in progress.

REFERENCES

(*1*) Gale, E. F., and Folkes, J. P., *Nature*, **26**, 1223 (1954)

(*2*) Nomura, M., *J. Agr. Chem. Soc. Japan.*, **29**, 674 (1955)

(*3*) Nomura, M., Maruo, B., and Akabori, S., J. Biocheme, **34,** 143 (1956)

(*4*) Hagihara, B., *Ann. Rep. Scient. Works*, Fac. Sci. Osaka Univ. **2**, 35 (1954)

(*5*) Nomura, M., unpublished experiments

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